CEFood & Congress

Novi Sad, Serbia
23 - 26 May, 2012

PROCEEDINGS

of 6th Central European Congress on Food

IUFoST International Union of Food Science and Technology

EFFoST European Federation of Food Science and Technology

CEI Central European Initiative

ISBN 978-86-7994-027-8

6TH CENTRAL EUROPEAN CONGRESS ON FOOD, Novi Sad 2012, SERBIA

Publisher

University of Novi Sad, Institute of Food Technology
Bulevar cara Lazara 1.
21000 Novi Sad, Serbia

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Cover
Boris Bartula, BIS, Novi Sad, Serbia

Printed by
“Futura” – Novi Sad, Serbia

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11. EDUCATION, INNOVATION AND KNOWLEDGE TRANSFER

FUNCTIONAL AND SENSORY PROPERTIES OF PASTA ENRICHED WITH LIGHT BUCKWHEAT FLOUR

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ABSTRACT: Standard wholegrain wheat flour pasta formulation was modified by replacement of wholegrain wheat flour with 20% of light buckwheat flour in order to improve functional properties of pasta. Content of free and bound phenolic compounds in dry and cooked pasta was determined by HPLC/DAD. Sample colour was measured using Minolta Chromameter CR-400. Sensory properties of dry and cooked pasta were evaluated by trained panelists using a 5-point category scale.
The results have demonstrated a decrease of about 44% in total phenolic compounds after cooking in comparison to dry pasta, while 8.37% of the total phenolic compounds from dry pasta were present in cooking water. Light buckwheat flour substitution led to a decrease of lightness (L^*), but did not significantly ($P < 0.05$) affect redness (a^*), yellowness (b^*) and hue angle (h) values. Scores for sensory properties were between 4.00-4.80 for dry, and 2.70-4.60 for cooked pasta, indicating the satisfactory pasta quality.
Key words*: functional properties, sensory properties, pasta, buckwheat flour, colour determination*

INTRODUCTION

Pasta is food product that is mainly produced by mixing durum wheat semolina and water. It is a low price product that is largery consumed all over the world and its consumption tends to increase. Since that common wheat pasta is low in protein and essential amino acids, as well as some vitamins and minerals (Heger and Frydrych, 1987) it is recognised as a good matrix for supplementation with various health beneficial supplements.
Buckwheat (*Fagopyrum esculentum* Moench) possesses a great potential for upgrading the functional properties of foods due to its high content of proteins, starch, and vitamins. Antioxidants in buckwheat, including flavonoids, phenolic acids, tannins, phytosterols and tocopherols play important role as anti-inflammatory and anticarcinogenic agents (Kreft et al., 2006; Lin et al., 2009). Among the other cereals and pseudocereals, buckwheat is the richest source of rutin (Vachirapatama et al., 2011) which is confirmed to reduce the fragility of blood vessels and can improve C vitamin absorption. Buckwheat also contains biologically important quercetin that is very effective in anti-tumour, antioxidant and anti-inflammatory activities in vitro systems (Cui and Wang, 2012). Rutin and quercetin were not found in other cereals and pseudocereals. Another functionality of buckwheat comes from its gluten-free characteristics making buckwheat suitable for the diet for celiac disease patients (Sedej et al., 2011).
In order to produce a functional pasta with nutritional benefits, light buckwheat flour has been used for supplementation of a part of wheat flour in pasta formulation. However, the addition of some functional ingredients could result in changes in the sensory properties which may lead to changes in consumers' acceptance (Glanz et al., 1998).
Apart from the evaluation of the cooking quality and sensory properties versus the control pasta made of wheat flour, the aim of this work was to analyze the content of free, bound and total phenolic compounds in dried and cooked buckwheat enriched pasta, as well as in its cooking water.

MATERIALS AND METHODS

Samples
Two types of pasta (spiral shape) (W - wholegrain wheat pasta and WB - pasta enriched with 20% of light buckwheat flour) were produced by using a pasta extruder (Ital past Mac 60 (Parma, Italy)) and pasta dryer (at 40 ºC for 13 h) (Ital past D200).

Pasta cooking quality
Optimal cooking time (OCT), cooking loss (CL) and volume increase (VI) were determined according to Pravilnik o metodama fizičkih I hemijskih analiza žita, mlinskih I pekarskih proizvoda, testenina I brzo smrznutih testa (Sl. list SFRJ, 74/88; Jambrec et al., 2011).

Colour determination
Colour of dry pasta samples was determined by a Minolta Chromameter (Model CR-400, Minolta Co., Osaka, Japan), with granular attachment CR-A50. Colour values were recorded for five randomly chosen types of pasta per batch and averaged. The results were expressed as lightness (L^*), redness-greenness (a^*), and yellowness-blueness (b^*), according to CIE $L^*a^*b^*$ system. Dry pasta samples were ground in 1095 **Knifetec** sample mill.

Extraction of free and bound phenolic compounds from dried and cooked pasta
Cooked pasta was dried at 45 ºC for 1 day before milling. Prior to analysis, pasta samples were milled using 1095 Knifetec mill. A portion of about 5 g of milled pasta was extracted in an ultrasonic bath at 40 ºC with 12.5 mL of ethanol/water (4:1 v/v) for 10 min. The supernatants were collected and evaporated in a Reacti-Therm I (Thermo Fisher Scientific Bellefonte, PA. U.S.A.) to dryness at 40 ºC.
The isolation of bound phenolic compounds required the residues left after the separation of supernatants to be submitted to alkali hydrolysis. Residues were refluxed with 50 mL methanol and 10 mL 50% KOH (in water) for 20 min. Hydrolizates were filtered and neutralized with hydrochloric acid. The final solutions were extracted three times with 50 mL ethyl acetate. The organic fractions were pooled and evaporated in a rotary evaporator to dryness at 40 ºC.
All extracts were dissolved in 5 mL methanol/1% formic acid (1:1) before analysis and filtered through 0.45 µm pore size PTFE filter (Rotilabo-Spritzenfilter 13 mm, Roth, Karlsruhe, Germany) before injection into the HPLC system. Until then extracts were stored at –18 ºC.

Extraction of phenolic compounds from cooking water
Cooking water was evaporated and dried at 130 ºC from 90 min. After that, dry matter from cooking water was extracted in an ultrasonic bath at 40 ºC with 10 mL ethanol/water (4:1 v/v) for 10 min. The supernatants were collected, evaporated at 40 ºC, and dissolved with 5 mL of methanol/formic acid before analysis. The extracts were stored at –18 ºC until use.

HPLC/DAD analysis
HPLC analysis was performed by using a liquid chromatograph (Agilent 1200 series), equipped with a diode array detector (DAD), on an Agilent, Eclipse XDB-C18, 1.8 µm, 4.6 x 50 mm column, at a flow-rate of 1.000 mL/min. A single rapid resolution, reverse phase HPLC method suitable for the determination of 17 phenolic compounds was developed and validated as previously reported (Mišan et al., 2011).

Sensory evaluation
Sensory evaluation was carried out on dried and cooked pasta samples by a panel of five trained assessors from the Institute of Food Technology, Novi Sad. Sensory properties of dry and cooked pasta samples were evaluated by a 5-point category scale (Jambrec et al., 2011; Pestorić et al., 2010). Sensory profiling was performed using a generic descriptive analysis technique, included the selected representative properties of pasta. Each mark was

described with words, using previously prepared standard cards (Tang, et al., 1999; Pestorić 2007; Pestorić, 2011).
All samples were coded with three random numbers and presented simultaneously among assessors. Dry pasta samples were presented on the plastic plates, while cooked pasta samples were presented in thermal plastic cups and served at room temperature within 15 minutes after cooking. Distilled water was used to clean the mouth and hands between samples.

Statistical analysis

Results reported in this study are the means of three measurements, except for the colour measurements where the results are the means of five repetitions. ANOVA and Duncan's multiple range test were used to compare means at 5% significance level by using statistical data analysis software system STATISTICA (StatSoft, Inc. (2008) data analysis software system, version 10.0. www.statsoft.com).

RESULTS AND DISCUSSION

Cooking quality of pasta

The obtained results of pasta cooking quality are shown in Table 1. Optimal cooking time (OCT) of W was longer than WB. Chillo et al. (2008) and Manthey et al. (2004) explained this phenomenon by physical disruption of the gluten matrix and overall lower density of pasta containing buckwheat flour. The lower density provided a path for water absorption into the structure which resulted in a significantly ($P < 0.05$) shorter cooking time and caused significant increase in cooking loss. The obtained results are in accordance with the findings of Alamprese et al. (2007) and Bilgiçli (2009), who reported that pasta with buckwheat flour had a significantly higher VI during cooking than wheat pasta. The authors explained it by the irregular structure of buckwheat starch granules, which contained more amorphous areas than those of wheat. However, our results showed that the volume increase for WB was not significant**,** probably due to the low substitution level in this pasta formulation.

Table 1. Cooking quality and colour values of pasta samples

	Cooking quality			Colour values			
	OCT (min)	**CL (% d.b.)**	**VI**	***L****	***a****	***b****	***h****
W	9.30 ± 0.30^{b}	7.18 ± 0.30^{a}	2.86 ± 0.40^{a}	75.34 ± 0.38^{b}	2.85 ± 0.15^{a}	14.77 ± 0.07^{a}	79.09 ± 0.54^{a}
WB	8.00 ± 0.20^{a}	10.00 ± 0.40^{b}	3.29 ± 0.10^{a}	73.54 ± 0.59^{a}	2.79 ± 0.16^{a}	14.82 ± 0.37^{a}	79.36 ± 0.33^{a}

Abbreviations used in table: OCT– optimal cooking time; CL – cooking loss; VI – volume increase
Values are means of three measurements of the cooking quality and five measurements of the colour values ± SD
Values with the different superscript within a column are significantly different ($P < 0.05$)

Colour determination

Every modification in pasta formulation results more or less in colour changes of the final product (Švec et al., 2008). The obtained results for the pasta colour indicated that fortification of pasta with light buckwheat flour significantly decreased ($P < 0.05$) lightness that is in accordance with previously published results of the other authors (McWatters et al., 2003; Singh and Mohamed, 2007; Sudha et al., 2007), who found that the incorporation of different composite ingredients may contribute to darker surface product (Table 1). In addition, the level of supplementation of the pasta did not significantly affect the other colour values.

Influence of pasta processing and cooking on the phenolic content

The light buckwheat flour was added into standard wholegrain wheat pasta formulation in order to improve functional properties, first of all to increase the content of phenolic compounds. Polyphenolic compounds are known to be relatively thermally stable, but their content can be significantly reduced in the final product as a result of different processes which occur during the production (Mišan et al., 2011). Thus, as a part of this study, the influence of cooking process on the content of polyphenolic compounds was investigated. Rutin, quercetin, catechin, ferulic and vanilic acid were determined by using HPLC/DAD analysis (Table 2). Buckwheat pasta had a majority of phenolic compounds present in a free form, as previously was stated by Hung and Morita (2008). Results sugested that rutin was mainly presented in free buckwheat fraction and cooking process decreased its content for about 8.50%. Since that recommended doses for rutin are in the range between 10-25 mg/day for adults (Filipčev et al., 2011), the consumption of 100 g of pasta supplemented with buckwheat flour at the tested level, would satisfy about 2-5% of daily dose. During pasta cooking, rutin from its bound form was converted into quercetin, as showed by the increase of quercetin content for about 20%. Ferulic acid, the main phenolic acid in wheat grain (Sedej et al., 2010), was dominantly presented in bound form, which is in accordance to many studies (Inglett et al., 2011; Adom and Liu, 2002; Clifford, 1999).

The results have demonstrated that 56% of total phenolic compounds tolerated the cooking process; 8.37% of the total phenolic compounds were dissolved in cooking water and 35.63% were degraded. Catechin demonstrated minimum tolerance to the cooking process with cooking loss of about 57%.

Table 2. Phenolic content in dry and cooked light buckwheat enriched pasta (c (mg/kg dw))

	Bound		**Free**	
Compound	**Dry**	**Cooked**	**Dry**	**Cooked**
Rutin	1.73 ± 0.26^{a}	1.49 ± 0.43^{a}	4.21 ± 0.35^{b}	3.85 ± 0.03^{a}
Quercetin	1.08 ± 0.08^{a}	1.29 ± 0.08^{b}	1.25 ± 0.70^{a}	1.14 ± 0.18^{a}
Ferulic Acid	46.51 ± 15.05^{a}	40.65 ± 8.39^{a}	5.19 ± 0.39^{b}	3.21 ± 0.14^{a}
Vanilic Acid	0.81 ± 0.22^{a}	0.65 ± 0.21^{a}	1.91 ± 0.18^{b}	1.07 ± 0.02^{a}
Catechin	33.15 ± 6.69^{b}	7.56 ± 0.26^{a}	107.59 ± 9.24^{b}	52.96 ± 1.64^{a}
Sum	83.28	51.64	120.45	62.23

Total (Free + Bound) Phenolic compouns

Compound	**Dry**	**Cooked**	**Cooking water**
Rutin	5.94 ± 0.31	5.34 ± 0.44	2.47 ± 0.83
Quercetin	2.33 ± 0.74	2.43 ± 0.11	1.18 ± 0.60
Ferulic Acid	51.70 ± 15.21	43.89 ± 8.35	2.86 ± 0.71
Vanilic Acid	2.72 ± 0.32	1.72 ± 0.22	2.06 ± 0.47
Catechin	140.74 ± 11.18	60.52 ± 1.78	1.51 ± 0.13
Sum	203.43	113.90	10.08

Values are means of three measurements ± SD
Different latter in the same row indicate significantly different values (P<0.05)

Sensory evaluation

Sensory quality of produced pasta was defined on the basis of evaluation for four properties of dry and seven properties of cooked samples are presented in Figure 1. The obtained scores by sensory evaluation (Figure 1) were between 4.00-4.80 for dry, and 2.70-4.60 for cooked pasta. Buckwheat flour addition in pasta formulation was not significantly ($P < 0.05$) affect the sensory properties, except the elasticity and firmness of cooked pasta which scores were significantly lower. On the whole, the pasta samples with addition of light

buckwheat flour had almost the overall quality scores statistically equal to that of the pasta samples made only of wholegrain wheat. Moreover, from the results, it was observed that the addition of light buckwheat flour determined a good sensory quality due to the presence of resistant starch that is less available and derived from buckwheat flour (Skrabanja et al., 2001). The results of firmness and elasticity are in accordance with the data obtained from Manthey et al. (2004), who found that with the increase from 0% to 30% of buckwheat flour in pasta manufacture there was an enhancement of the firmness and elasticity but when pasta was dried at higher temperature. In general, referring to the results of sensory quality of the pasta, 20% of light buckwheat flour may be used in pasta formulation without producing a negative impact on the sensory properties.

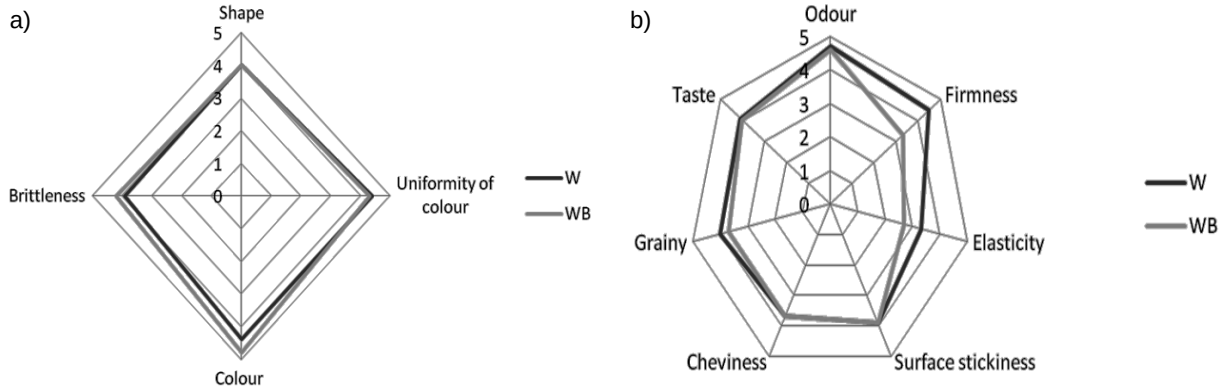

Figure 1. Sensory properties: a) dry pasta; b) cooked pasta

CONCLUSIONS

Supplementation of wholegrain wheat pasta formulation with 20% of light buckwheat flour induced a decrease in some pasta quality properties (significantly lower optimal cooking time and higher cooking loss). Modification of the pasta formulation resulted in darker pasta coloration (lower *L** values). Sensory evaluation showed that dried pasta with addition of light buckwheat flour demonstrated fairly similar sensory properties to the wholegrain wheat pasta. Analisys of the phenolic content showed that the cooking process negatively influenced the phenolic content. In general, in order to enhance functional properties, 20% of light buckwheat flour may be added into the wholegrain wheat pasta formulation without adversely affecting the sensory properties of the final product and may be regarded as health-promoting functional food.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31029 financed by the Ministry of Education and Science, Republic of Serbia.

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QUALITY AND ANTIOXIDANT CAPACITY OF NOVEL BEVERAGES BASED ON LEMON AND EXOTIC BERRIES

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ABSTRACT: Following previous research on lemon juice enriched with berries, the aim of this work was to design new blends based on lemon juice mixed with different edible berries from exotic and national origin: maqui (*Aristotelia chilensis* (Molina) Stuntz), açaí (*Euterpe oleracea* Mart.) and blackthorn (*Prunus spinosa* L.). The phytochemical characterization of controls and blends was performed by HPLC-DAD-ESI/MSn, and their antioxidant capacity against DPPH, superoxide and hydroxyl radicals and hypochlorous acid were also assessed. The profiling of the red fruits revealed a wide range of bioactive phenolics: anthocyanins, flavonols, 5-*O*-caffeoylquinic acid and ellagic acid derivatives. The lemon juice displayed flavones, flavanones, flavonols, and hydroxycinnamic acids. In a general way, the novel beverage based on lemon juice and maqui berry (LM) was the most interesting blend in terms of antioxidant capacity. The results suggested that lemon juice enriched with berries, mainly maqui, could be of potential interest in the design of new drinks with a nutritive related function on health for chronic diseases.
Key words: *Aristotelia chilensis, Euterpe oleracea, Prunus spinosa, Citrus limon, antioxidant*

INTRODUCTION

Nowadays, chronic diseases, including cancer, cardiovascular, and neurological disorders, have been taking special relevance in society. For this reason, a continuous flow of information and research results on the positive impact of fruits and vegetables on these diseases has been rising. Previous reports have demonstrated that berries have potential against chronic diseases, and their use in the fresh form or mixed with other juices could act as preventive and improve human´s health status in these disorders (González-Molina et al., 2008; Gironés-Vilaplana et al., 2012). In this sense, the design of new beverages combining lemon (*Citrus limon* (L.) Burm. f.) juice with berries, resulting in effectively increased antioxidant properties of the lemon juice, could offer new possibilities against this actual problem of chronic diseases.

Maqui (*Aristotelia chilensis* (Mol.) Stuntz) is a common edible berry from central and southern Chile that is a source of natural colorants due to the presence of anthocyanins. This fruit has also been recently reported as one of the healthiest berries, due to its bioactive components (Céspedes et al., 2008; Schreckinger et al., 2010). Several reports have linked maqui's phenolics with its high antioxidant capacity (Rubilar et al., 2011), *in vitro* inhibition of adipogenesis and inflammation (Schreckinger et al., 2010), protection against oxidative stress (Miranda-Rottmann et al., 2002), cardioprotection (Céspedes et al., 2008), and *in vitro* and *in vivo* anti-diabetic effects (Rojo et al., 2011; Rubilar et al., 2011). Açaí (*Euterpe oleracea* Mart.) is a berry from palm tree, which is native from Amazon River area in South America. It is commonly used fresh and in the preparation of beverages, and has recently become popular as a functional food due to its antioxidant potential and phytochemical composition (Lichtenthäler et al., 2005). Potential benefits have been attributed to açaí fruits, extracts and juices: antioxidant (Jensen et al., 2008), anti-inflammatory (Jensen et al., 2008), reduction of selected markers of metabolic disease risk (Udani et al., 2011) or atherosclerosis (Schauss et al., 2009), pain reduction improved mobility (Jensen et al.,

2011), and antiproliferative properties (Hogan et al., 2010). Blackthorn (*Prunus spinosa* L.) is a fruit of deciduous shrubs native to Europe, mainly Spain, Portugal and Turkey (Barros et al., 2010). It is commonly used in the preparation of jams or, in northeast Spain, macerated with aniseed liqueur to obtain a digestive alcoholic drink called patxarán. Fruits have also been used as astringent, diuretic and purgative (Barros et al., 2010), and have recently been proved as antioxidants (Ganhão et al., 2010).
The aims of this work were to perform a phytochemical characterization of lemon juice, maqui, açaí and blackthorn berries, to design new blends of lemon juice enriched with these berries (5% w/v), and to determine their antioxidant capacity for future applications in nutrition and health.

MATERIAL AND METHODS

Lyophilized fruits were added separately to lemon juice to obtain final concentration of 5% w/v of the fruit in the beverage. In addition, control solutions using the same proportion in 0.18 M citric acid buffer (pH 2.46) were prepared to study the activities of the different fruits without lemon. Lemon juice alone was also assayed. Samples were labelled as follows: L (lemon juice control), LM 5% (lemon juice plus 5% of maqui berry), M 5% (5% maqui in citric acid buffer), LA 5% (lemon juice plus 5% of açaí berry), A 5% (5% açaí in citric acid buffer), LB 5% (lemon juice plus 5% of blackthorn fruit), B 5% (5% blackthorn in citric acid buffer). All the methods were performed in triplicate.

HPLC-DAD-ESI/MSn

Chromatographic analyses were carried out on a Luna C18 column (250 x 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK). Water:formic acid (99:1, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 1 mL/min. The linear gradient started with 8% of solvent B, reaching 15% solvent B at 25 min, 22% at 55, and 40% at 60 min, which was maintained up to 70 min. The injection volume was 30 µL. Chromatograms were recorded at 280, 320, 360, and 520 nm. The HPLC-DAD-ESI/MS*n* analyses were carried out in an Agilent HPLC 1100 series equipped with a photodiode array detector and a mass detector in series (Agilent Technologies,Waldbronn, Germany). The HPLC system was controlled by ChemStation software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface and was controlled by LCMSD software (Agilent, version 4.1). The ionization conditions were adjusted at 350°C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from *m*/*z* 100 up to *m*/*z* 1200. Collision induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS*n* was carried out in the automatic mode on the more abundant fragment ion in MS(*n*-1).Prior to injection, samples were centrifuged (12000 rpm 5 min) and filtered through a PVDF Syringe Filter (0.22 µm). Anthocyanins were quantified as cyanidin 3-*O*-glucoside at 520 nm, flavonols as quercetin 3-*O*-rutinoside (rutin) at 360 nm, hydroxycinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm.

DPPH radical scavening activity

The antiradical capacity was estimated spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corporation), by monitoring the disappearance of $DPPH^{\bullet}$ at 515 nm, according to Oliveira et al. (2010).

Superoxide radical ($O_2^{\bullet-}$) scavenging activity

Antiradical activity was determined spectrophotometrically in a 96-well plate reader by monitoring the effect of controls and blends on the $O_2^{\bullet-}$ induced reduction of NBT at 560 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Ferreres et al., 2009).

Hypochlorous acid scavenging activity

The inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to DTNB was performed according to a described procedure (Valentão et al., 2002), in a double beam spectrophotometer (Heλios α, Unicam, Leeds, UK) at 412 nm.

Hydroxyl radical assay

The deoxyribose method for determining the scavenging effect of samples on hydroxyl radicals was performed as described before (Valentão et al., 2002) in a double beam spectrophotometer (Heλios α, Unicam, Leeds, UK), programmed in photometric function, with the wavelength fixed at 532 nm.

RESULTS AND DISCUSSION

The analysis of the berry fruits revealed the presence of a wide range of polyphenols. Concerning maqui, different glycosides and di-glycosides of delphinidin and cyanidin were found (A1-A6, A9 and A11) (Fig. 1 Table 1), in accordance to previous reports (Gironés-Vilaplana et al., 2012). Flavonols (quercetin and myricetin derivatives, F1-F5, F7-F10), ellagic acid derivatives (E2 and E3), 5-*O*-caffeoylquinic acid (C6) and one ellagitannin (granatin B, E1) were also identified (Fig. 1, Table 1). Only maqui sample presented ellagic acid derivatives. With respect to açaí, three derivatives of cyanidin (A7, A8, and A10) and one of malvidin (A14) were identified (Fig. 1, Table 1). Likewise, quercetin (F5, F7, F9, and F10) and hydroxycinnamic acid derivatives (C3, C6 and C8) were found too (Fig. 1, Table 1). These compounds were previously reported in açaí (Pacheco-Palencia, & Talcott, 2010). Blackthorn presented four anthocyanins (two cyanidin-glycosides (A8, and A10) and two peonidin-glycosides (A12, A13)) (Fig. 1, Table 1), quercetin derivatives (F4, F6 and F8), and hydroxycinnamic acid derivatives (C1-C5, and C7), (Fig. 1, Table 1), in accordance with previous researches (Ganhão et al., 2010). Lemon juice contained flavones, flavanones, flavonols, and hydroxycinnamic acids (Fig. 1, Table 1), as described before (González-Molina et al., 2008).

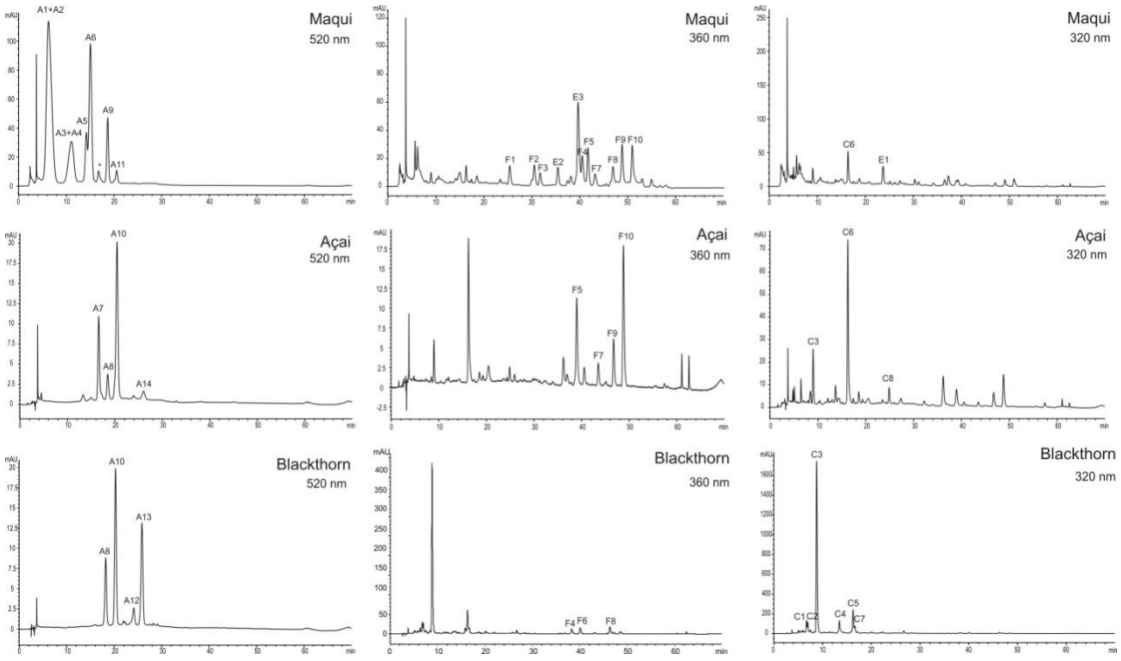

Figure 1. Chromatograms of berries registered at 520, 360 and 320 nm. The identities of the compounds associated with the peaks shown here are given in Table 1.

Table 1. Quantification (mg/100ml) of different phenolic compounds present in lemon juice control, berries controls, and blends.

Anthocyanins	**M 5%**	**A 5%**	**B 5%**	**LM 5%**	**LA 5%**	**LB 5%**
A1 Delphinidin 3-*O*-sambubioside-5-*O*-glucoside	16.66±0.37*	-	-	16.52±0.30*	-	-
A2 Delphinidin 3,5-*O*-diglucoside		-	-		-	-
A3 Cyanidin 3,5-*O*-diglucoside		-	-		-	-
A4 Cyanidin 3-*O*-sambubioside-5-*O*-glucoside	4.29±0.14 *	-	-	4.53±0.12	-	-
A5 Delphinidin 3-*O*-sambubioside	2.22±0.01	-	-	2.40±0.04	-	-
A6 Delphinidin 3-*O*-glucoside	7.41±0.07	-	-	7.44±0.13	-	-
A7 Cyanidin 3-*O*-galactoside	-	0.53±0.01	-	-	0.54±0.03	-
A8 Cyanidin 3-*O*-glucoside	-	0.18±0.00	0.70±0.09	-	0.17±0.00	0.64±0.01
A9 Cyanidin 3-*O*-sambubioside	2.36±0.03	-	-	2.49±0.04	-	-
A10 Cyanidin-3-*O*-rutinoside	-	1.26±0.02	1.52±0.16	-	1.11±0.06	1.42±0.04
A11 Cyanidin 3-*O*-glucoside-5-*O*-rhamnoside	0.52±0.00	-	-	0.58±0.01	-	-
A12 Peonidin 3-*O*-glucoside	-	-	0.24±0.03	-	-	0.22±0.00
A13 Peonidin 3-*O*-rutinoside	-	-	1.18±0.09	-	-	1.09±0.04
A14 Malvidin 3-*O*-glucoside	-	0.08±0.00	-	-	-	-
TOTAL ANTHOCYANINS	33.45±0.61	2.09±0.00	3.64±0.37	34.00±0.18	1.93±0.10	3.36±0.09
Non-coloured flavonoids	**M 5%**	**A 5%**	**B 5%**	**LM 5%**	**LA 5%**	**LB 5%**
Ellagic acid derivatives						
E1 Granatin B	6.35±0.19	-	-	6.57±0.83	-	-
E2 Ellagic acid hexoside	0.73±0.07	-	-	0.55±0.02	-	-
E3 Ellagic acid rhamnoside	3.73±0.75	-	-	2.27±0.19	-	-
TOTAL ELLAGIC ACID DERIVATIVES	10.81±0.63	-	-	9.40±1.00	-	-
Flavonols						
F1 Myricetin 3-*O*-galoylglucoside	1.22±0.14	-	-	0.71±0.02	-	-
F2 Myricetin 3-*O*-galactoside	1.46±0.19	-	-	1.17±0.04	-	-
F3 Myricetin 3-*O*-glucoside	0.84±0.10	-	-	0.45±0.03	-	-
F4 Quercetin 3-*O*-rutinoside	2.38±0.24	-	1.11±0.17	2.12±0.04	0.81±0.02	1.98±0.04
F5 Quercetin 3-*O*-galactoside	2.36±0.37	2.31±0.07	-	2.09±0.08	1.68±0.02	-
F6 Quercetin 3-*O*-hexoside-5-*O*-pentoside	-	-	1.21±0.15	-	-	1.38±0.05
F7 Quercetin 3-*O*-glucoside	0.89±0.11	0.50±0.04	-	0.76±0.05	0.54±0.07	-
F8 Quercetin 3-*O*-xyloside	0.45±0.02	-	1.52±0.21	0.60±0.01	-	1.51±0.01
F9 Quercetin 3-*O*-arabinoside	0.95±0.15	0.63±0.00	-	1.50±0.15	0.71±0.03	-
F10 Quercetin 3-*O*-rhamnoside	2.41±0.37	-	-	1.49±0.30	-	-
TOTAL FLAVONOLS	12.95±1.69	3.45±0.11	3.84±0.55	10.87±0.69	3.73 ± 0.13	4.88±0.09
Hydroxycinnamic acid derivatives (320 nm)						
C1 Caffeoyldihidrocaffeoylquinic acid (1)	-	-	1.96±0.25	-	-	1.83±0.01
C2 Caffeoyldihidrocaffeoylquinic acid (2)	-	-	1.78 ± 0.29	-	-	1.73±0.01
C3 3-*O*-caffeoylquinic acid	-	0.38±0.03	31.26±3.47	-	0.47±0.01	33.58±0.05
C4 3-*O*-*p*-coumarylquinic acid	-	-	3.42±0.45	-	-	3.15±0.41
C5 4-*O*-caffeoylquinic acid	-	-	4.42±0.60	-	-	4.04±0.14
C6 5-O-caffeoylquinic acid	1.07±0.02	1.36±0.19	-	1.58±0.04	2.01±0.02	0.45±0.06
C7 3-O-feruloylquinic acid	-	-	1.44±0.17	-	-	1.65±0.05
C8 5-O-*p*-coumaroylquinic acid	-	0.13±0.01	-	-	0.07±0.01	-
TOTAL CINNAMIC ACID DERIVATIVES	1.07±0.02	2.25±0.22	44.28±1.77	3.54±0.13	4.64±0.03	47.64±0.64

*Values are the mean ± standard deviation (n=3). M: 5% maqui in citric acid , A: 5% açaí in citric acid , B: 5% blackthorn in citric acid, LM: 5% maqui in lemon juice, LA: 5% açaí in lemon juice, LB: 5% blackthorn in lemon juice. nq= not quantified. *Anthocyanins A1 + A2 and A3 + A4 were quantified together.*

Concerning the different antioxidant methods, in DPPH$^{\bullet}$ the maqui samples displayed the strongest activity (Table 2). All the samples, controls, and beverages showed a similar strong effect against $O_2^{\bullet -}$, being the blend of maqui berries plus lemon juice (LM) the most interesting (Table 2). Regarding HOCl the activity was of lesser intensity, but again LM was the most active sample (Table 2). LM and the blend of açai berries and lemon juice exerted

the highest capacity against •OH (Table 2). Although samples reacted differently against radical depending on the antioxidant methods employed, it is remarkable that the novel beverage based on lemon juice and maqui berry (LM) was the most active in all the assays tested (Table 4), probably due to the contribution of high content of polyphenols to the blend. Nevertheless, the results suggested that the polyphenol content is not the only reason for the antioxidant potential, but also the quality and the interactions between compounds in the food matrix may be also involved (Sun et al., 2011).

Table 2. Antioxidant activity of control fruits and blends

IC_{50}	DPPH•	$O_2^{\bullet-}$	HOCl	•OH
L	13.27 ± 0.17d	5.24 ± 0.43e	25.71 ± 0.43d	6.93 ± 0.22d
M	9.06 ± 1.07bc	3.10 ± 0.13bc	40.49 ± 0.03f	4.34 ± 0.19c
A	18.91 ± 0.68e	3.78 ± 0.24cd	42.80 ± 0.29g	4.72 ± 0.21c
B	21.10 ± 0.58f	5.49 ± 0.19e	39.05 ± 0.63e	3.30 ± 0.09ab
LM	5.05 ± 0.14a	2.31 ± 0.14a	15.28 ± 0.06a	2.79 ± 0.08a
LA	7.39 ± 0.25b	4.42 ± 0.18d	18.84 ± 0.22b	2.85 ± 0.42a
LB	9.55 ± 0.83c	2.96 ± 0.30ab	21.90 ± 0.34c	3.49 ± 0.06b
LSD, p<0.05	0.51	0.20	0.29	0.17

Results are expressed in IC_{50} (n=3, mg/ml). L: Lemon juice, M: 5% maqui in citric acid, A: 5% açaí in citric acid, B: 5% blackthorn in citric acid, LM: 5% maqui in lemon juice, LA: 5% açaí in lemon juice, LB: 5% blackthorn in lemon juice. Means (n=3) in the same columns followed by different letters are significantly different at P < 0.05 according to Tukey's test.

CONCLUSIONS

New blends made of lemon juice and different exotic (maqui, açaí) and Iberian (blackthorn) berries with health promoting activities were developed, and their phytochemical profile has been described, revealing a wide range of bioactive phenolics: anthocyanins, flavonols, hydroxycinnamic acid derivatives and ellagic acid derivatives in berries and flavones, flavanones, flavonols, and hydroxycinnamic acids from lemon juice. Results of the different radical-scavenging methods indicated that the lemon-maqui novel beverage (LM) is the most interesting blend in terms of antioxidant activity.

ACKNOWLEDGEMENTS

Authors would like to express their gratitude to the Spanish Ministry of Economy and Competivity for the funding through the project CONSOLIDER-INGENIO 2010 Research Project FUN-C-FOOD (CSD2007-00063) and Fundação para a Ciência e a Tecnologia (FCT) for grant no. PEst-C/EQB/LA0006/2011. Part of this work was also funded by CYTED programme thematic network 112RT0460.

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EVALUATION OF PROTEIN AND LIPID CONTENT AND DETERMINATION OF FATTY ACID PROFILE IN SELECTED SPECIES OF CYANOBACTERIA

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ABSTRACT: Microalgal biomass is a rich source of some nutrients, such as n-3 and n-6 fatty acids, β-carotene, proteins, minerals, and other essential nutrients, which could potentially be used as functional food ingredients. In this work, the fatty acid composition and protein content of *Spirulina Platensis* and several non-toxic strains of *Nostoc spp*. and *Anabaena spp.* originating from Serbia was investigated. Analyses of fatty acid methyl esters (FAMES) were carried out by gas chromatography coupled with flame ionization detection (GC-FID), and the content of crude protein was determined by Dumas combustion method. The results show that 16 carbon (16:0 and 16:1 types) and 18 carbon (18:1, 18:2 and 18:3 types) chain fatty acids represent the most significant constituents of these species. Content of the relatively rare γ-linoleic acid (GLA, 18:3n-6) was highest in the *Spirulina* samples, while it is also shown to be present in the *Nostoc* species, depending on the chemical composition of the growth medium used. All of the investigated species showed very high crude protein content in the dry algal biomass (ranging from 42.8% to 76.5%). It can be concluded that the investigated species of cyanobacteria represent potential rich sources of protein and commercially attractive fatty acids and, since they are shown to be non-toxic, they can be considered as components of various functional food products.
Key words: *cyanobacteria, functional foods, fatty acids, protein*

INTRODUCTION

Cyanobacterial species are among the oldest living organisms known to exist today, and have been known to be used as human food since ancient times (Henrikson, 2009; Ciferri, 1983). In recent years, there has been an increased interest in benefits of using functional foods, that is, foods that are able to provide additional health benefits for human health, other than basic nutritional and energetic requirements (Goldberg, 1996). Microalgal biomass is a rich source of some nutrients, such as n-3 and n-6 fatty acids, β-carotene, proteins, minerals, and other essential nutrients, which could potentially be used as functional food ingredients. A number of papers investigating various potential health benefits of cyanobacteria exist today (Henrikson, 2009; Estrada et al., 2001; Tokusoglu and Unal, 2003). *Spirulina platensis*, which is known for its nutritional properties is a very rich source of biologically valuable proteins and essential fatty acids. Species of cyanobacteria which are especially rich in lipids can also be considered as a potential future source of biofuel and other industrial products (Mata et al., 2010).
In this work, the fatty acid composition and protein content of *Spirulina Platensis* and several non-toxic strains of *Nostoc spp*. and *Anabaena spp.* originating from Serbia was investigated. Preliminary results of the analyses are given in order to assess the potential use of these microorganisms as components of functional foods.

MATERIAL AND METHODS

Samples of *Spirulina Platensis*, *Nostoc spp*. (labeled as 2S7B) and *Anabaena spp*. (labeled as C2 and C5) strains were obtained from the Department of Biology and Ecology at the Faculty of Sciences, University of Novi Sad. All of the investigated strains originated from Vojvodina region of Serbia (Simeunović, 2005). *Nostoc* and *Anabaena* strains were cultivated under laboratory conditions in synthetic mineral broth BG-11, with (+N) and without added nitrogen (-N) (Rippka et al.,1979) while *Spirulina* strains were cultivated in mineral SOT broth (Soong, 1980). All the investigated cyanobacteria were cultivated as steady cultures in Erlenmeyer flasks at temperature of 22-24^{o}C and light intensity of 50 μmol m-2s-1. Daily light regimen was set to 12 hours of light and 12 hours of darkness. After 25 days of cultivation, the strains were lyophilized to obtain the dry algal biomass for analyses and kept well sealed in refrigerator until usage.

Protein and total lipid content

LECO Truspec CHNS analyzer (LECO, St. Joseph, MI, USA) which uses the Dumas combustion method for nitrogen determination was used for analysis of the lyophilized samples. Content of crude protein was calculated from the nitrogen content and expressed as the protein content of the dry algal biomass (Beljkaš et al., 2010).

Total lipid content was determined by extraction of the samples with chloroform-methanol solution (3×3 ml at 2:1 ratio of chloroform to methanol) and weighing the residue after evaporation of the solvents in the stream of nitrogen (Colla et al., 2007).

Fatty acid determination

Fatty acid methyl esters were prepared from the extracted lipids by transesterifitacion using 14% boron(III)-fluoride in methanol (Karlović and Andrić, 1996). The obtained samples were analyzed by a GC Agilent 7890A system with flame-ionization detector (FID), autoinjection module for liquid samples, equipped with fused silica capillary column (DB-WAX 30 m, 0.25 mm, 0.50 um). Helium was used as a carrier gas (purity > 99.9997 vol %, flow rate = 1.26 ml/min). The fatty acids peaks were identified by comparison of retention times with retention times of standards from Supelco 37 component fatty acid methyl ester mix (Sigma-Aldrich, EU) and with data from internal data library, based on previous experiments. Results were expressed as mass of fatty acid or fatty acid group (g) in 100 g of fatty acids.

RESULTS AND DISCUSSION

The results of the total lipid and protein content of the investigated samples are shown in Figure 1. The total lipid content varied from 6.83% (for C5+N *Anabaena spp.* strain) to 26.99% (*Spirulina Platensis*). Addition of nitrogen compounds to the cultivating broth negatively impacted the total lipid content of the investigated *Nostoc* and *Anabaena* strains, which is in accordance with similar findings by other authors (Tedesco and Duerr, 1989). The crude protein content was found to be very high in all the investigated cyanobacterial samples, varying from 42.8% (2S7B-N *Nostoc* spp. strain) to 76.5% (C5+N *Anabaena spp.* strain). In this case, the addition of nitrogen compounds to the cultivating broth positively impacted the protein content of the *Nostoc* and *Anabaena* strains. It should be noted however, that the Dumas combustion method which was used for determination of crude protein content causes all forms of nitrogen in the investigated sample to be converted to molecular nitrogen and therefore even the non-protein nitrogen containing compounds (alkaloids, amino acid derivatives, inorganic nitrogen compounds, etc.) can falsely be measured as total protein content (Beljkaš et al., 2010).

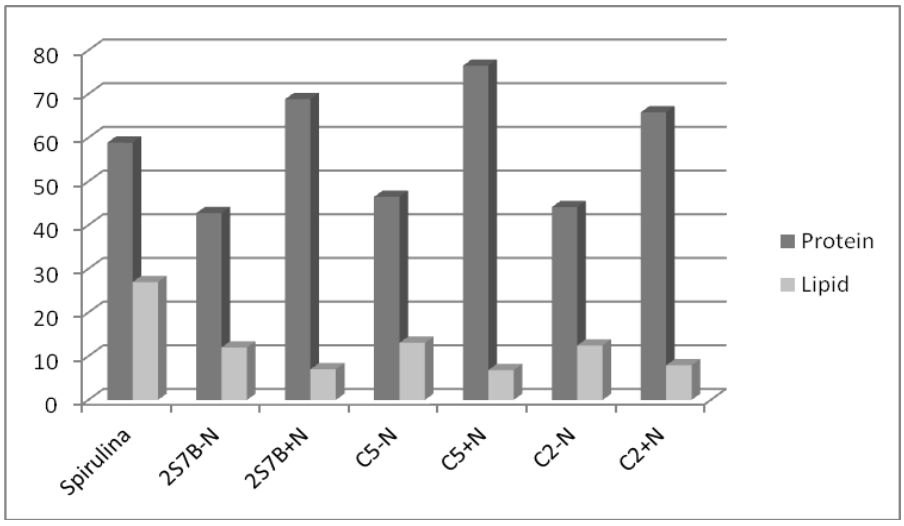

Figure 1. Total lipid and protein content of the investigated cyanobacterial strains

The results of fatty acid analysis by GC-FID are shown in table 1. The results show that 16 carbon (C16:0 and C16:1 types) and 18 carbon (C18:1, C18:2 and C18:3 types) chain fatty acids represent the most significant constituents of these species. Undecanoic acid (C11:0) acid was also determined in all of the investigated samples, ranging in content from 1.96% to 4.65%. Content of the relatively rare γ-linoleic acid (GLA, C18:3n-6) was highest in the *Spirulina* samples, while it is also shown to be present in the *Nostoc* species, although only in trace amounts (not shown in the table). *Spirulina* also showed a presence of significant amount (16.2%) of *cis*-11-eicosenoic (C20:1) acid, which was not present in other investigated species of cyanobacteria. *Nostoc* and *Anabaena* samples had a significant amount of α-linoleic (C18:3n-3) and arachidic (C20:0) acid, which were not present in the investigated *Spirulina* species, as well as much higher content of *cis*-13,16-docosadienoic (C22:2) acid (ranging from 6.80% to 13.07% in these species compared to 0.18% in *Spirulina* sample).

Saturated fatty acids (SFA) have been labeled as a possible cause of cancers and coronary heart disease when present in excessive amounts in human diet. The mean ratio of PUFA/SFA recommended by the British Department of Health is more than 0.45, and WHO/FAO experts have reported guidelines for a “balanced diet” in which suggested ratio of PUFA/SFA is above 0.4 (Wood et al., 2008; HMSO, 1994). All of the investigated species of cyanobacteria showed a favourable PUFA/SFA ratio (from 1.65 to 3.71).

Table 1. Fatty acid composition (% m/m) of the investigated cyanobacterial strains

fatty acid	Spirulina	2S7B-N	2S7B+N	C5-N	C5+N	C2-N	C2+N
4:0	1.02	0.00	0.00	0.00	0.00	0.00	0.00
6:0	0.00	0.03	0.03	0.26	0.04	0.02	0.03
11:0	3.29	4.65	4.40	2.89	2.49	3.46	1.96
12:0	0.02	0.00	0.00	0.02	0.01	0.01	0.00
13:0	0.17	0.54	0.93	0.53	0.89	1.64	2.29
14:0	0.03	0.13	0.00	0.09	0.13	0.13	0.10
14:1	0.80	1.09	1.11	0.23	0.20	0.22	0.16
16:0	9.28	6.47	5.49	5.30	5.22	6.61	5.48
16:1	14.98	39.99	35.06	49.54	43.26	42.31	35.91
17:0	0.52	1.65	1.18	0.52	0.45	2.15	1.68
17:1	0.12	0.19	0.28	0.14	0.11	0.00	0.20
18:0	0.31	0.36	0.44	0.27	0.37	0.28	0.43
18:1n9 t+c	0.90	2.39	4.04	1.38	2.01	1.62	2.90
18:2n6 t+c	15.86	13.49	17.21	9.37	11.17	9.29	12.80
18:3n6	34.69	0.00	0.00	0.00	0.00	0.00	0.00
18:3n3	0.00	14.27	11.03	16.47	15.24	18.73	14.96
20:0	0.00	5.83	4.87	4.93	3.75	4.84	5.38
20:1	16.22	0.00	0.00	0.00	0.00	0.00	0.00
20:2	0.55	0.18	0.33	0.13	0.13	0.13	0.18
21:0	0.13	0.00	0.00	0.00	0.13	0.00	0.00
20:4 n6	0.27	0.11	0.00	0.00	0.00	0.00	0.00
20:3 n3+n6	0.29	0.00	0.00	0.00	0.00	0.00	0.00
20:5 + 22:6	0.00	0.00	0.00	1.20	6.16	1.77	4.49
22:1n9	0.13	0.09	0.54	0.01	0.23	0.00	0.00
22:2	0.18	8.54	13.07	6.69	7.96	6.80	11.00
SFA	13.92	19.73	17.31	14.86	13.49	19.14	17.37
MUFA	32.35	43.76	39.92	51.30	45.60	44.15	39.01
PUFA	51.66	36.59	28.57	33.86	32.71	36.71	32.44
UFA	84.01	80.35	68.49	85.15	78.31	80.86	71.44
PUFA/SFA	3.71	1.85	1.65	2.28	2.42	1.91	1.87

SFA - saturated fatty acid; MUFA - monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; UFA - unsaturated fatty acids (total)

CONCLUSIONS

The investigated species of cyanobacteria in this work have shown to possess very high protein content, but further analysis of the amino acid composition is necessary in order to assess their potential value as a source of proteins in human diet. The fatty acid profile of all the investigated samples showed a favourable PUFA/SFA ratio which indicates a potentially positive health effect of the fats obtained from these species. It can be concluded that the investigated species of cyanobacteria represent potential rich sources of protein and commercially attractive fatty acids and, since they are shown to be non-toxic, they can be considered as components of various functional food products.

ACKNOWLEDGEMENTS

This paper is a result of the research within the Technological Development Project (TR-31029) supported by the Ministry of Education and Science, Republic of Serbia.

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THE INFLUENCE OF POLYPHENOLS ON GENERATION OF FREE RADICALS AS THE PRECURSORS OF MAILLARD REACTION PRODUCTS

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ABSTRACT: Thermally treated foods, like bakery products, contain Maillard reaction products some of which are proved as the substances with antioxidant properties. There are many literature data stating that Maillard reaction products together with polyphenols as the potent antioxidants contribute to the overall antioxidant capacity of some bakery products. It was found that pyrazine cation radicals are the precursors in formation of Maillard reaction products. The aim of this work was to investigate the interaction of these radicals with some polyphenols that are frequently contained in ingredients used for bakery production. The formation of relatively stable free radicals in alkaline reaction mixtures (pH 9.0) of the model systems consist of D(+)-glucose (1.0 M) and structural isomers of aminobutanoic acid (1.0 M) heated at the temperature of 98 °C for 20 minutes has been detected by electron spin resonance (ESR) spectrometry. Based on the analyses of hyperfine structure of ESR spectra of reaction mixtures of model systems, the presence of 1,4-disubstituted pyrazine cation radicals formed at the initial stage of Maillard reaction was found. Different polyphenols (gallic, caffeic, ferulic, chlorogenic acid, and tannic acid) were added in model systems to investigate their influence on the generation of pyrazine cation radicals. Decrease of relative intensities (RI) of ESR spectra signals indicated polyphenols' ability to inhibit the generation of the pyrazine cation radicals. It was found that tannic acid was the most efficient of all investigated polyphenols in inhibiting the formation of free radicals at the initial stage of Maillard reaction followed by gallic $>$ caffeic $\geq$ chlorogenic $>$ ferulic acid. These findings indicate a complex relation between antioxidants and the precursor of Maillard reaction products. Further investigation is needed to reveal the overall contribution of both polyphenol(s) and Maillard reaction products to antioxidant capacity of thermally treated product.
Key words: *pyrazine cation radicals, polyphenols, ESR*

INTRODUCTION

Browning reactions, which are some of the most important reactions occurring during food processing and storage represent the phenomenon with significant implications in food technology, nutrition and health. The major groups of reactions resulting in browning are enzymatic and non-enzymatic browning. The latter is favoured by thermal treatment and includes different reactions such as Maillard reaction and caramelisation.
Maillard reaction occurs between the reactive carbonyl group of the sugar and the amino group of the amino acids to produce compounds responsible for a range of odours and flavours in thermally treated foods.
It has been reported that Maillard reaction products possess certain antioxidant activity (Dittrich et al., 2003; Morales and Jimenez-Perez, 2001; Yoshimura et al., 1997). The highest antioxidant activity of Maillard reaction products is generally associated to the formation of melanoidins (Hayase et al., 1989; Yen and Hsieh, 1995; Manzocco et al., 2001). Therefore, increased antioxidant activity of some functional bakery products was addressed not only to the presence of polyphenols present in the functional ingredients but to the antioxidant activity of Maillard reaction products which were formed during baking (Hsu at al., 2004).

From that point of view, Maillard reaction is desirable for increasing overall antioxidant capacity of thermally treated products.
On the other hand, Maillard reaction is known for the formation of relatively stable free radicals in the initial stage of the reaction which were identified as 1,4-disubstituted pyrazine cation radicals (Namiki and Hayashi, 1975; Hayashi et al., 1977; Hayashi and Namiki, 1986; Milić and Piletić, 1984). Kato et al. (1996) found that the reaction mixture of glucose and glycine in the initial stage of Maillard reaction showed characteristic multiline electron spin resonance (ESR) signals due to the presence of 1,4-di(carboxymethyl)pyrazine cation radical.
Pyrazine cation radical may be a key intermediate of imidazoquinoxaline-type mutagens in heated foods (Felton and Knize, 1991; Kikugawa et al., 2000), especially meat (Pearsons et al., 1992).
Food antioxidants, ascorbic acid and erythorbic acid effectively scavenged the pyrazine cation radical generated in the reaction of glucose and glycine (Kikugawa et al., 2000). It could be scavenged by phenolic antioxidants including epigallocatehin gallate (Kato et al., 1996), cysteine and unsaturated fatty acids (Kikugawa et al., 1999). Furthermore, heterocyclic amines as the genotoxic carcinogens that were produced via pyrazine cation radical formation were affected by the addition of black or green tea, and of the tea polyphenols theaflavine gallate and epigallocatechin gallate (Weisburger et al., 1994). Two flavonoids, luteiolin and quercetin, and caffeic acid were found to suppress the formation of heterocyclic amines in cooked foods (Oguri et al., 1998).
Knowing that Maillard reaction may increase overall antioxidant activity of thermally treated foods and also provides pyrazine cation radicals that could deplete antioxidants, the aim of this work was directed to the investigation of the interaction of free radicals characteristic for Maillard rection with some polyphenols frequently contained in ingredients which are used inbakery production.

MATERIAL AND METHODS

Model systems

The formation of relatively stable free radicals in the initial stage of the Maillard reaction was carried out in following model systems:

I D(+)-glucose (1.0 M) - 2-aminobutanoic acid (1.0 M)
II D(+)-glucose (1.0 M) - 3-aminobutanoic acid (1.0 M)
III D(+)-glucose (1.0 M) - 4-aminobutanoic acid (1.0 M)

The pH value of model systems was adjusted to 9.0 using 0.1 M potassium hydroxide and obtained mixtures were heated at the temperature of 98 °C.
The initial stage of the Maillard reaction was detected by measuring the formation of relatively stable free radicals after 20 minutes (blank probe).
The influence of different polyphenols (gallic, caffeic, ferulic, chlorogenic acid, and tannic acid) on the formation of free radicals which are characteristic for the initial stage of the Maillard reaction was investigated by adding each polyphenol at the concentrations of 10^{-3}, $5x10^{-3}$, 10^{-2}, $2.5x10^{-2}$, $5x10^{-2}$ and 10^{-1} mol/dm^3 to the blank probe before heating.
Antiradical activity (AA in %) of poliphenols was calculated according to the following equation:

$$AA=100\cdot(h_o-h_x)/h_o \qquad /1/$$

where h_o and h_x are the heights of the first peak in the ESR spectrum of free radicals of blank probe and probe, respectively.

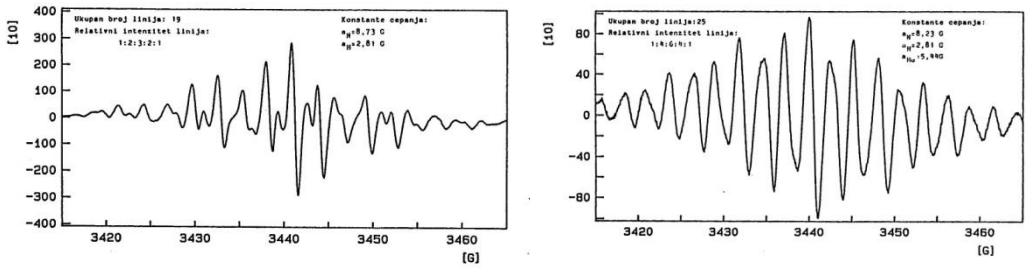
Detection of free radicals

ESR spectra of generated free radicals in investigated Maillard reaction model systems were recorded using Bruker 300E ESR spectrometer (Bruker, Rheinstetten, Germany) under the following conditions: modulation field 100 kHz, modulation amplitude 1.021 G, receiver gain

10^3, time constant 1.28 ms, conversion time 5.234 s, centre field 3440.00 G, sweep width 50.00 G, microwave frequency 9.65 GHz, microwave power 0.632 mW, and temperature (23 ± 1) °C.
A quartz flat cell Bruker ER-160FC was used for detection. Splitting constants were calculated from computer-generated second derivatives of the spectra after optimizing signal-to-noise ratios and were verified by computer simulations. ESR spectral files were imported into the WinSim program (WinSim, Sugar Land, TX, USA) for the analysis of the hyperfine splitting constants (Duling, 1994).

DISCUSSION AND RESULTS

The formation of relatively stable free radicals in alkaline reaction mixtures (pH 9.0) of the model systems consist of D(+)-glucose (1.0 M) and structural isomers of aminobutanoic acid (1.0 M) heated at the temperature of 98 °C for 20 minutes has been detected by electron spin resonance (ESR) spectrometry. The ESR spectra of produced radicals in the initial stage of Maillard reaction are presented in Fig. 1a and b.
The ESR spectrum presents in Fig. 1a consists of 19 lines of the relative intensities of 1:2:3:2:1, characteristic for the presence of 1,4-disubstituted pyrazine cation radicals formed in both systems with the hyperfine coupling constants of a_N = 8.73 G and a_H = 2.81 G. After computer simulation of the ESR spectrum with these hyperfine coupling constants, the obtained simulated spectrum had the same appearance as the experimental spectrum.
In contrast to model systems I and II, ESR spectrum registered in model system III (Fig. 1b) is characterized by hyperfine coupling constants of two pyrazine nitrogens (a_N = 8.23 G), four equivalent pyrazine protons (a_H = 2.81 G) and four equivalent side chain protons (a_H = 5,44 G) confirming the formation of 1,4-disubstituted pyrazine cation radicals in the model system III.
These observations were consistent with the earlier results of Namiki and Hayashi (1975), Milić and Piletić (1984) and Rizzi (2004).
The addition of polyphenols in the concentration range from 10^{-3} to 10^{-1} mol/dm^{-3} in all investigated model systems (blank probes) affected the formation of 1,4-disubstituted pyrazine cation radicals. It was registered by decreasing of intensities of ESR signal.
The influence of gallic, caffeic, ferulic, chlorogenic acid, and tannic acid on the generation of pyrazine cation radicals are presented in Fig 2.



a) b)

Fig. 1. ESR spectra of reaction mixture (a) of model systems I and II (2- or 3-aminobutanoic acid (1.0 M) and D(+)-glucose (1.0 M)) heated at 98 °C for 20 minutes; (b) of model system III (4-aminobutanoic acid (1.0 M) and D(+)-glucose (1.0 M)) heated at 98 °C for 20 minutes

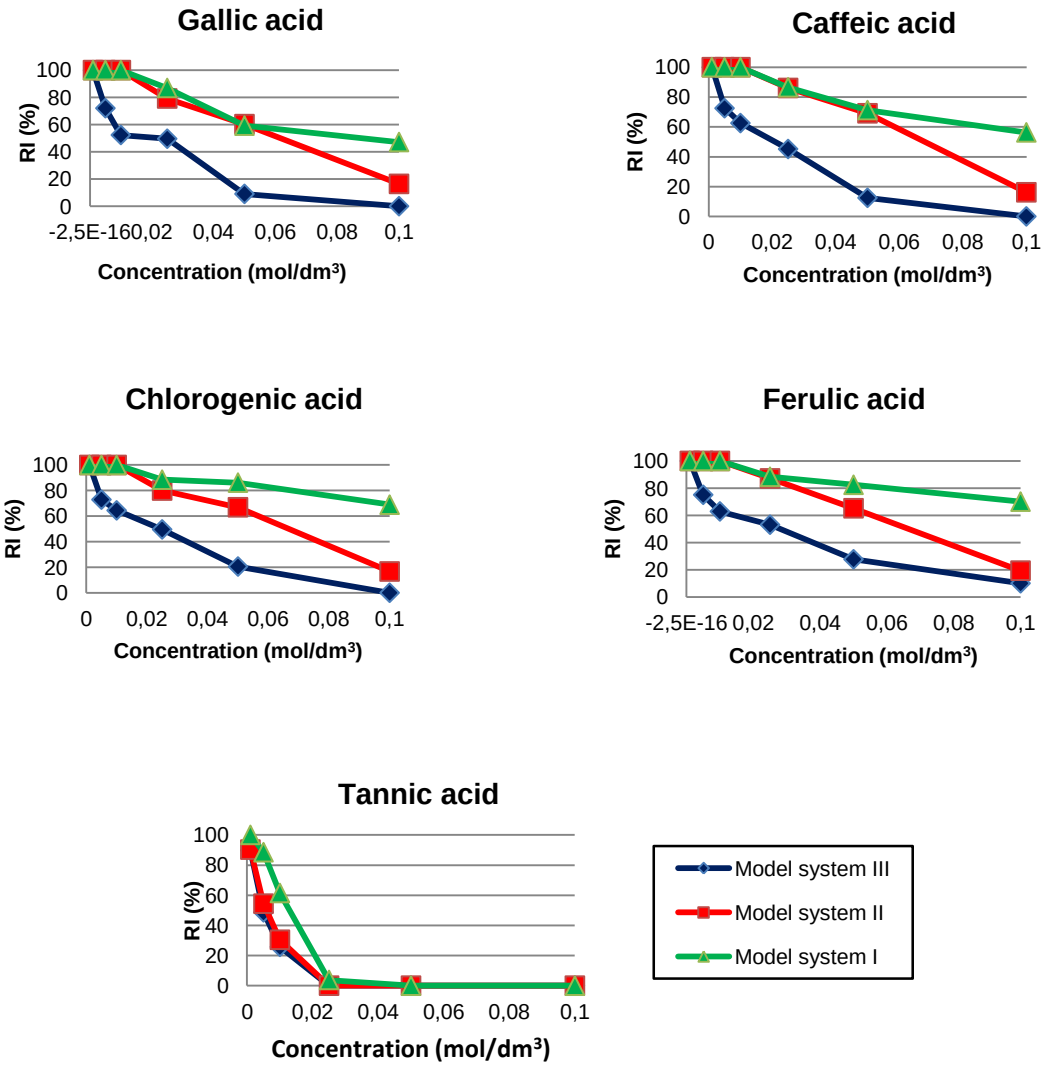


Fig. 2. The influence of (a) gallic, (b) caffeic, (c) ferulic, (d) chlorogenic acid, and (e) tannic acid on the generation of 1,4-disubstituted pyrazine cation radicals formed in the initial stage of the Maillard reaction in model systems I, II and III

Phenolic compounds which are known as powerful antioxidants (Shahidi and Wanasundara, 1992) probably inhibit the generation of pyrazine cation radicals in alkaline media by a single electron transfer followed by conversion of phenolic compounds into phenoxyl radicals. The stabilization of phenoxyl radicals by resonance depends on the structure of antioxidants and influences their effectiveness. The proposed mechanism could be confirmed by the results which were obtained using quinic acid in the defined concentration range (10^{-3}-10^{-1} mol/dm^{-3}) to suppress the formation of pyrazine cation radicals in all model systems. It was found that quinic acid, the moiety of chlorogenic acid, did not inhibit the formation of pyrazine cation radicals under all investigated conditions. That means that chlorogenic acid achieved its antiradical activity towards pyrazine cation radicals through its caffeoyl part via proposed mechanism.

The antiradical activity of polyphenols depends on the model system, as well as the type and concentration of polyphenols (Fig. 2).

The effectiveness of investigated polyphenols to suppress the formation of pyrazine cation radicals in investigated model systems was in the following order: model system III > model system II > model system I. The established order could be explained by steric hindrance between pyrazine cation radical and polyphenol. Namely, pyrazine cation radical formed in the model system III possess four α-protones in contrast to two α-protones bound to the

pyrazine cation radical generated in model systems I and II. Therefore, the interaction between pyrazine cation radical and polyphenol in model system III could be easier and resulted in better suppression of formation of pyrazine cation radicals.
It was found that tannic acid was the most efficient of all investigated polyphenols in inhibiting the formation of free radicals in the initial stage of Maillard reaction followed by gallic $>$ caffeic $\geq$ chlorogenic $>$ ferulic acid. Effectiveness of investigated acids is the result of the abilities of formed phenoxyl radicals to stabilize by resonance. It was previously reported that gallic acid is much potent antioxidant that caffeic and chlorogenic acids due to its chemical features (Brand-Williams et al., 1995). Similar antiradical activities of caffeic and chlorogenic acids were due to their similar structures, i.e. chlorogenic acid reacts using its caffeoyl part as it is mentioned above.
Ferulic acid expressed the lowest antiradical activity towards pyrazine cation radicals among all investigated polyphenols, but was still active (Fig. 3). As this compound represents the major phenolic acid in many cereal products (Liyana-Pathirana and Shahidi, 2007), its activity is very important, especially in bakery products.
Tannic acid was identified as the most potent inhibitor in formation of pyrazine cation radicals (Fig. 3). The ability of tannic acid to act as a potent suppressor was supported by the stability of tannic acid radicals which were detected by ESR (Fujita at al., 1988).
The involvement of polyphenols in the inhibition of formation of pyrazine cation radicals in the initial stage of Maillard reaction leads to the dilemma about contribution of Maillard reaction in overall antioxidant activity of thermally treated bakery products. Nevertheless, it is doubtless that polyphenols could scavenge pyrazine cation radicals and inhibit the formation of imidazoquinoxaline-type mutagens in heated foods.

CONCLUSIONS

It was found that polyphenols inhibit the formation of pyrazine cation radicals that were formed in the initial stage of Maillard reaction in heated alkaline reaction mixtures of model systems consisted of D(+)-glucose and structural isomers of aminobutanoic acid. The effectiveness of investigated polyphenols to suppress the formation of pyrazine cation radicals was in the following order: tannic $>>$ gallic $>$ caffeic $\geq$ chlorogenic $>$ ferulic acid.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31029 “Funkcionalni proizvodi na bazi žita namenjeni osobama sa metaboličkim poremećajima (Functional Cereal-based Products for People with Metabolic Disorder)” financed by the Ministry of Education and Science, Republic of Serbia.

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NATURAL EXTRACTS FROM *PTEROSPARTUM TRIDENTATUM* AT DIFFERENT VEGETATIVE STAGES: EXTRACTION YIELD, PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY

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ABSTRACT: The aerial parts of *Pterospartum tridentatum*, a wild growing species in Portugal used in traditional medicine and gastronomy, were harvested at different stages (vegetative phase, flowering phase and beginning of dormancy) in two locations in Portugal (Malcata and Gardunha mountains), and the respective aqueous extracts have been studied. The influence of the seasonal variation in the extraction yield, total phenolic content and antioxidant activity was evaluated. The extraction was carried out in boiling water in consecutive steps. After each step, the aqueous extract was separated and fresh water was added maintaining the same plant material. The procedure was repeated seven times, within an overall time period of 180 minutes.
Higher extraction yields were achieved with plant stems collected at the vegetative phases, either from Malcata or Gardunha regions. The total phenolic content of the extracts from Malcata plants ranged from 273 mg to 400 mg gallic acid equivalent/g dry matter, which was quite similar to that determined for extracts from Gardunha (245 to 394 mg gallic acid equivalent/g dry matter). The antioxidant activity was determined by the radical scavenging activity method using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). The greatest radical scavenging activity was observed in the flowers extracts, even though all extracts produced presented a good antioxidant activity. Furthermore, the antioxidant activity was not affected by the exposure of the plant material at 100ºC for long periods of time (180 min).
The results show that *Pterospartum tridentatum* has a great potential to be used as a new source of natural antioxidants for the food industry.
Key words: *Pterospartum tridentatum; Aqueous extracts; Extraction yield; Antioxidant activity; Phenolic content.*

INTRODUCTION

Researchers are looking for natural antioxidants as alternative to synthetic compounds, such as butylated hydroxytoluene (BHT), butylated hidroxyanisole (BHA) and *ter*-butylhidroxyquinone (TBHQ) that are widely used in the food and pharmaceutical industries. The interest in plant-derived food additives has grown, because the consumption of synthetic antioxidants has been related to the possible health risks.
Some plants synthesize secondary metabolites with biological activity, namely poliphenolic compounds and alkaloids. The amount and type of metabolites produced is dependent on the plant species and environmental factors that affect growth.
Polyphenols participate in plant defense mechanisms to counteract reactive oxygen species in order to avoid oxidative damage. They are often presumed to be safe for human consumption, due to their plant origin, and are used as health promoting ingredients (Moyo *et al*., 2010).
Pterospartum tridentatum L. Willk. [=*Chamaespartium tridentatum* (L.) P. Gibbs.; *Genista tridentata* L. is an European endemic Leguminosae (=Fabaceae) species belonging to the subfamily Papilionoideae and known as *carqueja* or *carqueija* in Portugal. This small shrub,

growing spontaneously up to 100 cm, is very common in the mountains of the north of Portugal and can usually be found in the understory of *Arbutus unedo, Pinus pinaster* and *Eucalyptus* forests. Some authors refer the use of *P. tridentatum* in popular medicine, claiming to possess digestive properties and activity against high blood pressure, cholesterol, diabetes and even obesity (Vitor *et al*., 2004). It is also used in gastronomy as a condiment in rice and rabbit stew to improve flavor. *Carqueja* is an underexploited natural source of compounds with biological activity, which should be fully characterized aiming to its valorization.
In the present study, the aerial parts of *P. tridentatum* were collected in two locations in Portugal (Malcata and Gardunha mountains), at three different vegetative stages. The effect of the harvest period and extraction time on the extract yield, as well as on the total phenolic content and antioxidant activity, was evaluated.

MATERIAL AND METHODS

Samples of the aerial parts of *Pterospartum tridentatum* were collected at different stages: vegetative phase (end of February), flowering period (in May) and beginning of dormancy (end of October). The shrubs were collected in two locations in Portugal: Gardunha and Malcata mountains.
Consecutive aqueous extraction steps were performed by refluxing a mixture of 25 g of plant samples and 100 mL of distilled water in a Clevenger apparatus. They were processed in consecutive time periods in boiling water: 15, 30, 60, 90, and 120, 150 and 180 minutes. After each time period, the aqueous phase was removed and the extraction continued after the addition of an equal amount of fresh water to the same plant material. The recovered aqueous phases were freeze-dried and a solid extract was obtained.
The total phenolic content (TP) of the extracts was evaluated by the method Ribéreau-Gayon (1970), measuring the absorbance at 280 nm. TP values were expressed as mg gallic acid equivalents per gram dry matter. All trials were carried out in duplicate.
The antioxidant activity of the solid extracts was determined by the radical scavenging activity method using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). The freeze-dried extracts (0.2 mg) were dissolved in water (1 mL), and added to a methanolic DPPH solution (4 mL). After 40 min incubation period at room temperature, in the dark, the absorvance was measured at 517 nm. The radical scavenging activity (RSA) was calculated as follows: RSA (%) = $[(Abs_{control} - Abs_{sample})/ Abs_{control}] \times 100$, where $Abs_{control}$ is the absorvance of DPPH radical in methanol and Abs_{sample} is the absorvance of DPPH radical after reaction with sample extract. Results were expressed as Trolox equivalents (mM Trolox/100 g extract dry mass).

RESULTS AND DISCUSSION

Extraction yield

The cumulative extraction yields presented some differences when comparing different harvesting seasons. Higher extraction yields were achieved from plant steams collected at the vegetative phases, either from Malcata or Gardunha regions (Figure 1). Regarding the extraction from plant parts collected in Malcata, a higher extraction yield was also obtained from the flowers (Figure 1A), when compared to that obtained from stems collected during flowering and in the beginning of dormancy. For all vegetative stages, there was an increase of the cumulative extraction yield over the consecutive batch extraction steps, for all the collected plant parts. This fact means that the plant material still contained unextracted compounds even after seven extraction steps.

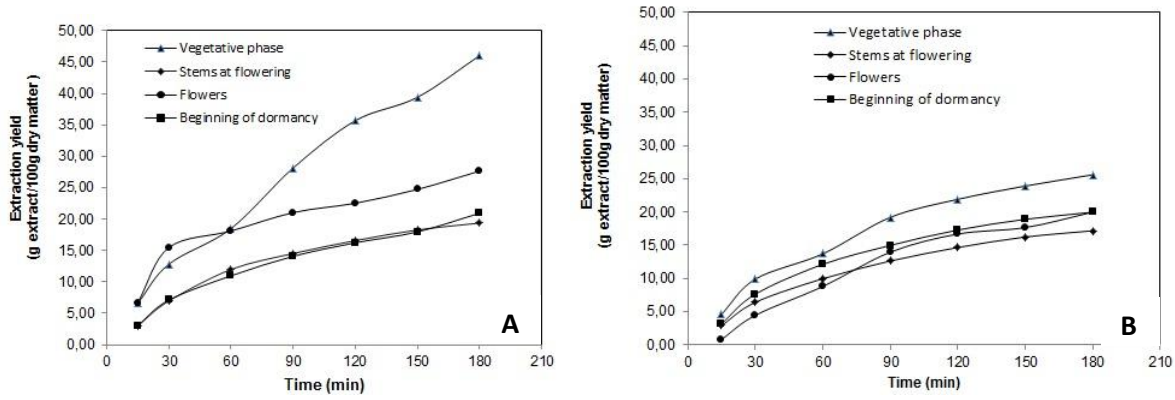

Figure 1. Cumulative curve of the extraction yield of *P. tridentatum* aereal parts collected in Malcata (A) and Gardunha (B) at various stages (stems at the vegetative phase; stems during flowering; flowers; stems in the beginning of dormancy).

The stems from flowering phase and beginning of dormancy showed lower yield values comparing to the plant parts harvested in the other phases. In the beginning of dormancy this fact may be explained by the plant investment in the previous period with fruiting and seed production. The lower extraction yield observed with stems from flowering phase may be due to the channeling of reserves for the floral organ.

In order to determine the most convenient extraction time (which is related with the number of batch extraction steps), the mass of extract recovered in each extraction step was converted as the percentage of the overall mass of extract obtained (Table 1). It was found that the mass of extract recovered after 60 minutes (three extraction steps), for Malcata and Gardunha plants, was between 40 and 65% of the overall mass recovered. After 90 minutes (four extraction steps) the percentage of extract recovered was between 61% to 79%, and after 120 minutes (seven extraction steps) it was on average more than 75%, which may be considered an acceptable value for the extraction time and energy costs involved. In most cases, the addition of an extra hour of extraction leads to a low increase on the cumulative extraction yield. For that reason, the extraction time of 120 minutes (five extraction steps) was considered to be a good compromise between the amount of extract recovered and the time and energy involved in the process.

Table 1. Mass of extract as percentage of the overall mass recovered as a function of extraction time.

	Malcata				**Gardunha**			
Time (min)	**Extract recovered (%)**				**Extract recovered (%)**			
	Vegetative phase	Flowering		Beginning dormancy	Vegetative phase	Flowering		Beginning dormancy
		Stem	Flower			Stem	Flower	
15	14.4	15.6	24.2	14.7	17.9	16.7	4.5	15.9
30	27.9	36.0	55.9	34.0	38.6	37.2	25.1	38.1
60	40.2	61.9	65.5	52.4	53.5	57.9	49.7	60.7
90	61.1	75.0	76.1	67.3	74.9	73.5	79.0	75.0
120	77.5	85.6	81.5	77.6	85.5	85.4	94.5	86.6
150	85.6	94.4	89.5	85.8	93.4	94.6	100.0	94.7
180	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

According to the results obtained by Luis *et al*. (2009) it is verified that the values of extraction yield of aqueous extracts in *Erica* spp. and *Cytisus scoparius* was 4.64 and 4.98% w/w (d.m.) respectively. In another study including *Carissa opaca* species (medicinal product

used in Pakistan) the extraction efficiency was 10.7 ± 0.99% w/w (d.m.) (Sahreen *et al*., 2010). These values are much lower than those observed in this work.

Total phenolic content

The total phenolic content of the extracts recovered from Malcata plants (Figure 2A) ranged from 273 mg to 400 mg gallic acid equivalent/g dry matter, which was quite similar to that determined for extracts from Gardunha (245 to 394 mg gallic acid equivalent/g dry matter) (Figure 2B). For both Malcata and Gardunha plant extracts, the lowest value was observed in flowers extracts after 180 minutes of extraction (eight consecutive extraction steps) and the highest value in vegetative phase after 150 minutes.

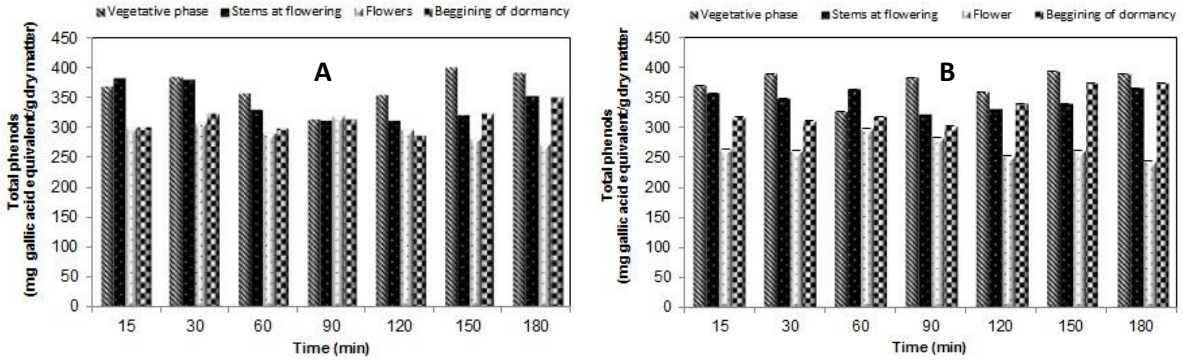

Figure 2 - Total phenols in the aqueous extract of *P. tridentatum* from Malcata (A) and Gardunha (B) (Vegetative phase; Stems at flowering; Flowers; Beginning of dormancy). Measurements were made in duplicate (SD ≤ 0.08).

We can consider that all extracts, from different vegetative phases and different extraction times, presented high levels of phenolic compounds when compared to other species described by Sousa *et al.* (2007). The phenols present in the species *Pterospartum tridentatum* show high levels at any stage of the vegetative cycle and were superior to other studied species such as *Harpephyllum caffrum*, *Sclerocarya birrea* (Ajila *et al*., 2010) and *Carissa opaca* (Saherr *et al*., 2010).

A similar study with five medicinal plants (*Terminalia brasiliensis*, *Terminalia fagifolia*, *Cenostigma macrophyllum*, *Qualea grandiflora*, *Copernicia prunifer)* carried out by Sousa *et al*. (2007) has shown much lower phenolic contents ranging from 11.55 mg gallic acid equivalent/g dry matter in the root extract of *C. prunifera* and 97.6 mg gallic acid equivalent/g dry matter in the leaf extract of *T. fagifolia*. Rockenboch *et al*. (2008) obtained values of total phenolic compounds in aqueous extract of *Physalis peruviana* fruit (47.8 mg gallic acid equivalent/g dry matter).

Antioxidant Activity

All extracts presented antioxidant activity, but higher AA values were obtained for the extracts recovered from plants harvested during the flowering period (Figure 3).

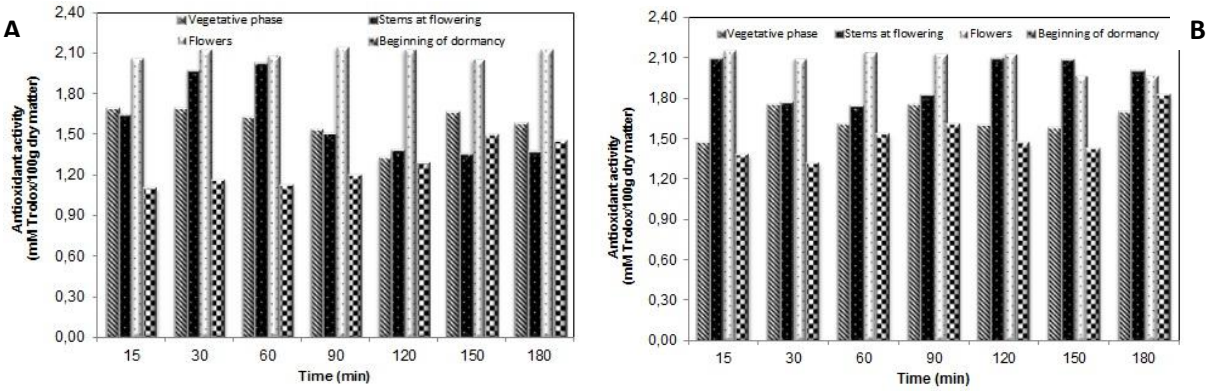

Figure 3 - Antioxidant activity of aqueous extracts of *P. tridentatum* from Malcata (A) and Gardunha (B) (Vegetative phase; Stems at flowering; Flowers; Beginning of dormancy). Measurements were made in triplicate (SD ≤ 0.02).

The values are consistent with the work reported by Luis *et al*. (2009), in which the AA of aqueous extract of *P. tridentatum* was 1.30 mMTrolox/100g dry matter.

CONCLUSIONS

The extraction process of *Pterospartum tridentatum* aerial parts presented a high extraction yield, being the highest yields achieved when processing flowers and plant stems harvested at the vegetative phase. The aqueous extracts presented high contents of total phenolic compounds, which did not change significantly with the harvesting season. In addition, consecutive extracts isolated from the same plant material over time have shown to possess similar total phenolic content and maintained their antioxidant activity. The results show that *Pterospartum tridentatum* has a great potential to be used as a new source of natural antioxidants for the food industry. Future studies will be focused on the identification of the major compounds present in the aqueous extracts and the correlation between the chemical species with the antioxidant activity. Vitor *et al*. (2004) identified some compounds such as isoflavones (namely prunetin, genistin and sissotrin) and the flavonol glycoside isoquercitrin.

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THE CONTENT AND RADICAL SCAVENGING CAPACITY OF PHENOLIC COMPOUNDS FROM BLACK RADISH ROOTS OF VARIOUS SIZES

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ABSTRACT:. In this paper content of phenolic compounds from black radish (*Raphanus sativus* L. var niger) roots of different size were examined. Black radish grown in Serbia, with the same period of development, grouped by size into three groups: (R1) with mass of 350±15 g; (R2): 100±10 g and (R3): 35±5 g, were used. Plant extracts were prepared by using 80% (v/v) ethanol and total phenolic compounds content was determined by spectrometric and the free radical scavenging abilities by DPPH radical method. The phenolic compounds content was in range 42.9 (R1) to 19.7 (R3) μmol chlorogenic acid per g of dried plant material, i.e. 443.7 to 208.6 μmol chlorogenic acid per g of dry extract. The radical scavenging capacity ranged from 88.3 (R1) to 55.6% (R3) and the EC_{50} values were from 1.59 to 2.24 mg/ml. The phenolic compounds content and radical scavenging capacity depended on root size in such a way that bigger root means higher content of phenolic compounds and higher scavenging capacity. By statistical analysis, there was a positive correlation between the phenolic compound content and radical scavenging capacity and Euclidean linkages distances results showed higher similarity between R2 and R3 sample.
Key words: *phenolic compounds, radish, scavenging capacity*

INTRODUCTION

Black radish (*Raphanus sativus* L. var*. niger*) is a plant belonging to *Cruciferae* family. In folk medicine, black radish root has been used since antiquity as a natural drug against the flatulence, formation of gallstones and indigestion (Hänsel, 1985; Pahlow, 1989). Black radish is an effective natural drug for the stimulation of bile function (Böhm, 1959; Ritter, 1984; Weiss, 1985) and significant antiurolithiatic effects of black radish were also reported (Vargas et al.1999). Black radish root contains mixture of various inorganic and organic compounds (minerals, lipids, proteins, carbohydrates, dietary fibres and vitamins).
The biological properties such as anticancer, antimicrobial and antiviral derive from phenolic compounds and sulphur and nitrogen containing organic compounds, such as glucosinolates (Lugasi et. al., 1998). Due to these abilities glucosinolates and phenolic compounds have great attention today. The glucosinolates occur as secondary metabolites of almost all plants of the order Brassicales, derived from glucose and oftenly two amino acids are incorporated. Plants use substances derived from glucosinolates as natural pesticides and as defence against herbivores. These substances are also responsible for bitter or sharp taste of radish (Volder et al., 2009).
Phenolic compounds are product of secondary metabolism of plants which arise biogenetically from the main synthetic pathways: shikimate and the acetate pathway (Bravo, 1998). Phenolic compounds have free radical radical scavenging abilities, which depend on the exposure to stress such as light, temperature, water (Nakamura et al., 2003; Kays et al., 1997), nutritional deficiencies (Dioxin et al., 1995; Robins et al., 1997) type of vegetable tissue (Sonia et al., 2007), mechanical damage such as wounding (Fernando et al., 2007), maturation stages (Ozgen et al., 2009), chemical structure (Lugasi et al., 2003) etc.

With regard to the widespread use of black radish in the diet in this paper the effect of the size of the root on the content of phenolic compounds composition and radical scavenging capacity (SC) were examined. The results could be good indicator for the consumers which size i.e. weight of black radish root for nutritive or healthy benefits is better to consume.

MATERIAL AND METHODS

Plant material

Black radish (*Raphanus sativus* **L**.) var. *niger*) roots, with the same period of development, based on root size i.e. weight, were grouped into R1 (350±15 g), R2 (100±10 g) and R3 (35±5 g) groups. Five samples of roots were collected for each group. The roots were cut in cube shape (1.5x1.5x1.5 cm), dried at 35 ^{o}C during 6 h and left at room temperature for 1 h. Then the five samples were mixed into appropriate group and milled to average particle size of 0.5 mm.Three separate determinations for each group were performed.

Plant material moisture content

The black radish roots moisture content was determined by using the analyzer (Scaltec SMO 01, Scaltec Instruments, Göttingen, Germany). Fresh plant material (3 g) was dried at 110 ^{o}C to a constant weight, and moisture content was read out on the analyzer display.

Extracts preparation

Plant extracts preparation for measurements of the phenolic compounds content were done according to Dokhani et al. (2005) procedure. Dried and milled black radish roots sample (3 g) was measured and 80 mL of 80% (v/v) ethanol was added. The mixture was stirred by MR1 magnetic stirrer (IKA-Werke, Staufen, Germany) for 10 minutes at 200 min^{-1} and vacuum filtered through No. 54 Wathman filter paper (GE Healthcare, Brondby, Denmark). The solids were re-extracted with 60 mL of 80% (v/v) ethanol, the filtrates combined and made to a final volume of 250 mL. For the measurement of phenolic compounds content, 10 mL of each extract was filtered through a 0.45 μm membrane filter (Agilent Technologies, Wilimington, Delaware, USA). For radical scavenging capacity (SC) measurements and HPLC analysis, 200 mL of each extract was evaporated in vacuum at 45 ^{o}C until dry and dissolved in 10 mL of 96% (v/v) ethanol.

Phenolic compounds content

For phenolic compounds content (PCC) measurement, a standard curve for five concentrations of chlorogenic acid (Sigma Chemical, St. Louis, Missouri, USA) concentrations, covering the range from 50 to 1500 μmol, was made. In a test tube, 0.25 mL of 0.1% (w/v) HCl in 95% (v/v) ethanol, 4.50 mL of 2% (w/v) HCl and 0.25 mL of chlorogenic acid standard solutions were added, mixed by vortex and allowed to stand for approximately 15 min. Then the absorbance (*A*) was read at 280 nm using UV 21 000 Sprectrophotometer (Cole Parmer Instruments Company, Vernon Hills, Illinois, USA) as described by Glories (1998). Based on standard curve, the equation for PCC determination was obtained as:

$$\text{PCC} = \frac{A - 0.1083}{4.890 \times 10^{-4}} \quad (1)$$

For measuring phenolic compounds content in black radish extracts, into test tube 0.25 mL of 0.1% HCl in 95% (v/v) ethanol, 4.50 mL of 2% (w/v) HCl and 0.25 mL of filtered extracts were added and further treated as standard solutions of chlorogenic acid. The PCC was obtained based on equation (1) and presented as μmol of chlorogenic acid per g of dry plant material and μmol of chlorogenic acid per g of dry extract.

Radical scavenging capacity

The radical scavenging capacity (SC) of extract diluted by ethanol to concentrations ranging from 0.2 to 6 mg/mL, was determined by the DPPH test (Mensor et al., 2001). Ethanol solution of DPPH radicals, 1 mL of a 0.3 mM, was added to 2.5 mL ethanol solution of given

concentration of investigated extract and allowed to react at room temperature during 30 min. Then the *A* value was measured at 518 nm on UV 21 000 Sprectrophotometer (Cole Parmer Instruments Company), and converted into the percentage of radical SC by using the equation (Mensor et al., 2001):

$$SC = 100 - \frac{(A_{sample} - A_{blank})}{A_{control}} \times 100 \quad (2)$$

where A_{sample} is the absorbance at 518 nm of the ethanol solution of the extract treated with the DPPH radical solution; A_{blank} is absorbance at 518 nm of the ethanol solution of the extract (1 mL of ethanol added to 2.5 mL of extract), and $A_{control}$ is absorbance at 518 nm of ethanol solution of DPPH radical (1 mL of a 0.3 mM/L added to 2.5 mL of ethanol). The final results are presented as EC_{50} value, i.e. concentration of investigated extracts sufficient to decrease the initial DPPH concentration by 50%.

Statistical analysis

Statistica version 5.0 software (StatSoft, Tulsa, Oklahoma, USA) was used to perform the statistical analysis: the mean, standard deviations and the correlation coefficients. The mean and standard deviations were obtained by Descriptive Statistics, marking the Median & Quartiles and Confirm Limits for Means and the correlation coefficients were obtained by correlations matrix analysis with displaying p and N value. The Euclidean distances were obtained by the cluster analysis and the Euclidean method with the complete linkage.

RESULTS AND DISCUSSION

Extract yield, phenolic compounds content and scavenging capacity

The results of moisture content, extract yield, the PCC and radical SC of phenolic extracts from black radish roots are shown in table 1. The results are mean values of three determinations. The dependence of total scavenging capacity on concentration of phenolic compounds content in extract, are presented in figure 1.

The results showed that the PCC varied in range from 42.9 (R1) to 19.7 (R3) μmol chlorogenic acid per g of dried plant material, i.e. 443.7 to 208.6 μmol chlorogenic acid per g of dry extract. Based on results presented on figure 1, the radical scavenging capacity ranged from 88.3 (R1) to 55.6% (R3) and the appropriate EC_{50} values were 1.59 and 2.24 mg/ml, respectively.

Table 1. Moisture content, extract yield, phenolic compounds content and radical scavenging capacity in black radish roots of various weights

Group of parsnip roots	R1 (350±15 g)	R2 (100±10 g)	R3 (35±5 g)
Fresh plant material moisture content (%)	68.2±1.9	71.9±1.3	70.8 ±1.6
Extract yield (g/kg)	48.2±3.5	47.1±3.6	46.4±3.4
Phenolic compounds content (μmol/g of dry plant material)	42.9±2.6	23.1±1.8	19.7±1.6
Phenolic compounds content (μmol/g of dry extract)	443.7±15	241.3±12	208.6±16
EC_{50} (mg/mL)	1.59±0.4	2.98±0.5	3.54±0.5

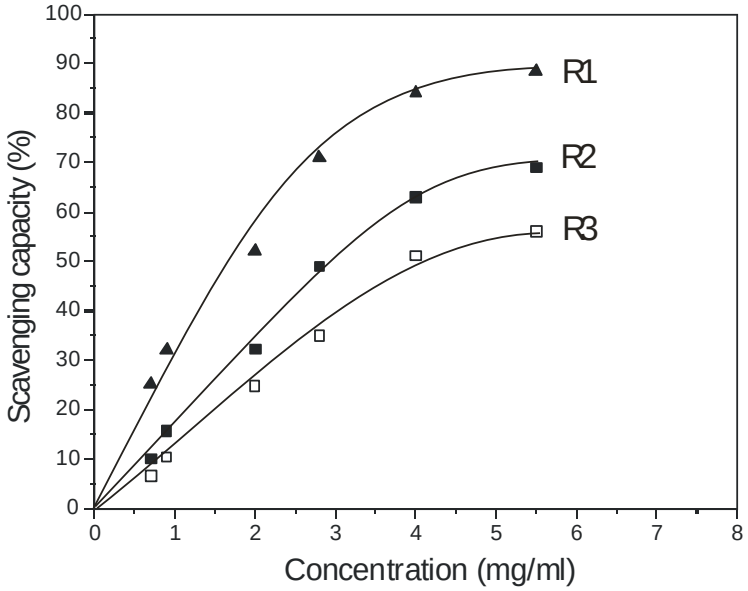

Figure 1. Total scavenging capacity of 80% (v/v) ethanol extract from black radish root of various sizes

Statistical analysis

The correlation coefficients were obtained based on five parameters (1- extract yield, EY; 2- phenolic compounds content as μmol of chlorogenic acid per g of dry plant material, PPC1; 3. phenolic compounds content as μmol of chlorogenic acid per g of dry extract, PPC2; 4- radical scavenging capacity, SC and 5- concentration of investigated extract sufficient to decrease the initial DPPH concentration by 50%, EC_{50}).

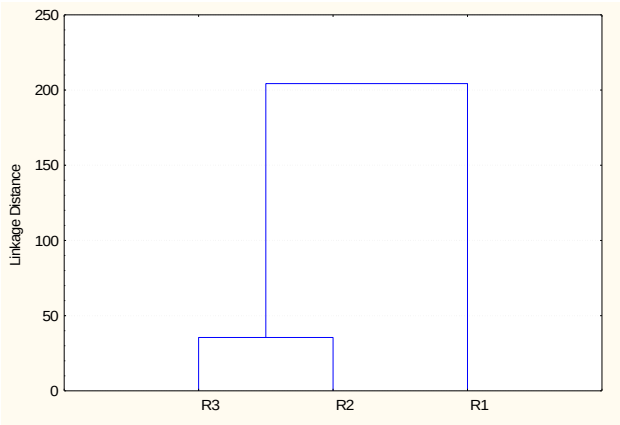

Figure 2. Total scavenging capacity of 80% (v/v) ethanol extract from black radish root of various sizes

The sample size was six (*N* = 6, three groups and their minimum and maximum value of determinations). The results show the high PCC was associated with high (the correlation coefficient was 0.96) and low EC_{50} values (the correlation coefficient was -0.99). As the lower EC_{50} value indicates higher antioxidant capacity, the obtained correlations show that higher phenolic compounds content in black radish root means higher scavenging capacity.

The results of Euclidean linkages distances based on the same parameters as the obtained correlation coefficients, showed higher similarity between R2 and R3 samples than between others (Figure 2), and this is evident from data in Table 1 and figure 1.

CONCLUSIONS

The phenolic compounds content and radical scavenging capacity depended on black radish root size in such a way roots with higher weight had higher content of phenolic compounds and better antioxidant capacity. There was a positive correlation between the phenolic compounds content and total radical scavenging capacity. The black radish root with weight higher than 100 g are recommended for human nutrition for healthy benefits.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project Grant No.172047 „Prirodni proizvodi biljaka i lišajeva: izolovanje, identifikacija, biološka aktivnost i primena" (Natural products from plants and lichens: isolation, identification, biological activity- the possibility for final application) by the Ministry of Education and Sciences Republic of Serbia.

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ANTIOXIDANT ACTIVITY OF THE SPECIES *AGROCYBE AEGERITA* IN RELATION TO TOTAL PHENOLIC (FLAVONOID) CONTENT

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ABSTRACT: In the last couple decades inedible polypore fungal species became of great importance as sources of new natural bioactive substances expressing various medical effects: antioxidative, anticancer, antimicrobial, and others. Recently, some edible mushroom species were also detected for their high bioactive potentials which are mostly influenced by the origin of the species. The species *Agrocybe aegerita* (Brig.) Sing., (syn. *Agrocybe cylindracea, syn. Pholiota aegerita)* is an edible, medium-size species broadly distributed in our region, mostly on poplar wood (lignicolous species). It is white-rot fungi containing many bioactive metabolites such as indole derivatives with free radical cavenging activity, cylindan with anticancer activity and agrocybenine with antifungal activity. Aiming to discover antioxidative activity of this indigenous fungal species, crude ethanolic extract from wild-growing species originated from Novi Sad (Serbia) was analyzed for DPPH free radical scavenging capacity (RSC) and total redox potential (FRAP assay)_in relation to total phenol (Folin–Ciocalteu assay) and total flavonoid content. RSC and total redox potential of extracts were in direct positive correlation with total phenol content. *A. aegerita* showed excellent RSC activity reaching EC_{50}≈28.88μg/ml in DPPH assay, the total phenol content (21.58±5.70 mg gallic acid equivalents/g of d.w.) and total flavonoid content (0.84±0.16 mg equivalent of quercetine/g d.w) and the FRAP value (13.47±2.64 mg ascorbic acid eq. mg/gd.w.). The HPLC determination showed that analyzed species contain caffeic, gallic, protocatechic and vanilic acids. Demonstrated results suggest that analyzed fungus contain phenolics that directly contribute to expressed activities and could be valuable sources of natural antioxidants in nutrition.
Key words: *antioxidative activity, extracts, radical scavenging capacity, phenolic acid, Agrocybe aegerita, total redox potential*

INTRODUCTION

Fungi have been used throughout mankind as the primary source of delicacy food called "food of gods" because of their specific aroma and taste. Throughout history they have been also known to possess medicinal effects such as anticancer, antidiabetic or immunomodulating, recognizing them as a great natural source of drugs. Due to their ability to produce a various nutritive and bioactive substances, including antioxidants (Lorenzen and Anke, 1998; Abraham, 2001; Lindequist et al., 2005, Elmastas et al., 2007; Ribeiro et al., 2007; Turkoglu et al., 2007; Dubost et al., 2007; Barros et al., 2007) fungi represent, beside plants, one of the most important sources for the development of new drugs. The scientific interest for fungal biomolecules as sources of antioxidants has grown recently (Mau et al., 2002; Lakshmi et al., 2004; Acharya, et al., 2004). However, the chemical nature of antioxidant compounds is still under investigation world over. Many authors assigned phenol compounds as the major antioxidants of some medicinal fungi (Mau et al., 2002; Cui, Kim, & Park, 2005), despite the fact that there are few data indicating that raw mushrooms represent a poor source of flavonoids and other phenols (USDA Database for the Flavonoid Content of Selected Foods 2003). It is well established that the content of total phenols could contribute to the antioxidant potential of food such as fruits and vegetables. Although fungi are not plants, previous studies that have analyzed the antioxidant properties of mushrooms have

shown a positive correlation between polyphenol content and antioxidant capacity (Kim et al., 2008; Karaman, 2010).
Agrocybe aegerita (V. Brig.) Singer, 1949 (fam. Bolbitiaceae) is known as black poplar or south poplar mushroom (English), like Albarelle, Aloumère (French), Yangimatsutake (Japan) or Zhuzhuang-Tiantougou (Chinese). It is broadly distributed species in South Europe, North America, India and China. It grows from April to October as parasitic or saprtotrophic species on weak or dead wood (poplar, elm, willow, ash). *A. aegerita* is an edible and very tasty species that has a mellow and attractive flavor when young and it is believed that this was the first cultivated mushroom in Europe. It grows in groups, on the smooth white shanks that reach a height of 10 cm. Traditionally it was used against headaches, dizziness, nausea and as a diuretic and antidiuretic in some parts of China (Giovaninni et al., 2006). Two substances showing antioxidant activity were isolated from the methanolic extract inhibiting lipid peroxidation in rat liver microsomes (Giovaninni et al., 2006). Phenolic compounds with low molecular weight and relatively high polarity from *Agrocybe aegerita* were positively correlated with scavenging radical activity and inhibition of LDL oxidation (Lo and Cheung 2005). From this fungus 1,3-βD-glucan has been isolated, showing activity against sarcoma 180 of mice while intraperitoneal administration of polysaccharides isolated from the species *A. cylindracea* has also shown activity in normal and streptomycin-induced diabetic mice (Kiho et al., 1994). A bioactivity-guided investigation detected ceramide, methyl-β-D-glucopyranoside and α-D-glucopyranoside, along with already reported linoleic acid and its methyl ester. Ceramide expressed COX and tumor cell proliferation inhibitory activities (Diyabalanage et al., 2009). According to a broad distribution of this species in our region and its high bioactivity known from the literature, the objective of this work was to investigate the composition and antioxidant activity of ethanolic extract of this indigenous edible fungal species by two biochemical tests in vitro: DPPH free radical scavenging capacity (RSC) and total redox potential. Total phenol and total flavonoid content of extract was measured due to point to the influence of these compounds on the express activities. Moreover, determination of the main components in extract was measured by HPLC chromatography.

MATERIALS AND METHODS

Fungal material and extraction

Wild growing lignicolous fungal species was collected from Sunčani kej location (Novi Sad) near Danube river on dead poplar's log in September, 2005. Samples of mature fruiting bodies were brush cleaned, air dried to constant mass and pulverized in an electrical mill. 10g of each sporocarp powder was extracted by stirring with 350 ml of 80% ethanol at 25ºC on rotary shaker (120 rpm) for 72 h and filtered twice (through absorbent cotton and filter paper). Extract were rotary evaporated at 40ºC to dryness. Dried extract were dissolved in the same solvent to a concentration of 10 %, and stored at 4ºC for further use.

DPPH assay

10 μl of fungal extract (5.0-500 μg/ml) was mixed with 1 ml of methanol solution containing $DPPH^{\bullet}$ (Sigma) to obtain a final concentration of solution of 90 μM $DPPH^{\bullet}$, and filled up with pure MeOH to 4 ml. The mixture was shaken vigorously and left for 60 minutes in the dark and then the absorbance was measured at 515 nm (Soler-Rivas, et al., 2000). The reactions were carried out in triplicate and recorded against tert-butylated hydroxytoluene – BHT (0.25 mol/dm^3) (Fluka AG; Buchs, Switzerland) and butylated hydroxyanisole –BHA (0.32 mol/dm^3) as a positive control. The $DPPH^{\bullet}$ scavenging activity was calculated as a RSC (radical scavenging capacity) by using the following equation:
$RSC\ (\%)=100x\ (A_{blank}-A_{sample}/A_{blank})$.
The concentrations of the extract that express 50 % neutralization of DPPH radical (EC_{50} values), were determined by linear regression analysis from the obtained RSC values in software Origin 2001.

FRAP assay
Assay of ferric reducing ability of the extract is based on comparing the change in absorbance at 600 nm of a sample with the change in absorbance of a known standard ($FeSO_4.7H_2O$) to detect level of antioxidant activity (Griffin and Bhagooli, 2004). This procedure involves the reduction of Fe^{III}-TPTZ to a blue colored Fe^{II}-TPTZ by any biological antioxidant or chemical reductants. Ascorbic acid was used to calculate the standard curve.

Total phenol and flavonoid content
The total phenol content of fungal extract was expressed as gallic acid equivalents (GAE) per g of dry extract (d.w.). It was measured according to slightly modified method using a Folin-Ciocalteu reagent (Fukumoto and Mazza, 2000). After 2h, absorbance was read at 760 nm. Total flavonoid content was measured using a method based on formation of a complex flavonoid-aluminium (Park *et al.*, 1997). The absorbance was determined at 415 nm after incubation (30min, 25ºC). Total flavonoid concentration was calculated using quercetin (Q) as standard, and the results were expressed in mg quercetin equivalents (QE) per g of dry extract (d.w.).

HPLC determination of phenolic acids
Chromatographic analysis of phenolic acids was carried out by HPLC according to the method described previously (Bendini *et al.*, 2003). The HPLC equipment consisted of an integrated system with a pump HP 1100 (Agilent Technologies, Palo Alto, USA) with stationary phase C18 (5 µm particle size, 150 × 4.5 mm I.D. Flow, USA) and UV-DAD detector (280 nm). The mobile phase used was a mixture of two components (95% A + 5% B), where A was a mixture of twice-distilled water and acetic acid (98:2, v/v) and B was a mixture of methanol and acetonitrile (1:1, v/v) at a flow-rate of 0.5 mL/min and elution with linear gradient. Standards of phenolic acids were dissolved in the mobile phase (95% A + 5% B). The compounds in each sample were identified by comparing their retention times and UV-VIS spectra with the standards.

RESULTS AND DISCUSSION

Extraction yield and total phenolic (TP) and flavonoid content (F) in the analyzed extract are presented in Table 1. *A. aegerita* had three times higher average yield (Table 1) than other analyzed species prepared in the same solvent reaching approximatelly 4% (Karaman, 2009). At the same this extract showed the highest solubility. Thus it can be concluded that this edible species is suitable for application in the form of alcoholic tinctures. Obtained results were in accordance with the results established for ethanol extracts of *A. aegerita* species from Istra region in Slovenia (Mujić et al., 2010) when compared both the final yield of extract and total phenol content (15.52%, 23.07 mg/g GAE, respectively). Yield of fungal extract was slightly higher for Slovenian species than for species from Novi Sad what can be explained by the polarity of solvents applied reaching slightly higher yield with 50% EtOH than with 80% EtOH. Moreover, TP/TF ratio was much higher in Slovenian species (21.85%) than the ratio obtained in this work. This can be explained by the fact that different standard equivalent (catechine) was used for calculations of standard curve. However, total phenolic content for Chinese *Agrocybe* species (Ng and Cheung, 2004) contained three times higher content of total phenols than analyzed species.

Table 1. Extraction yield and total phenolic (TP) and flavonoid content (F) in fungal extract

Fungal species	Yield of extract[a] (g/10g) d.w.	Extraction (w/w) %	Total phenols[b] (mg/g)	Flavonoids[c] (mg/g)	F/TP[d] %
A. aegerita	1.19±0.20	**11.93**	21.58±5.70	0.84±0.16	3.9

[a]Extracted from dried fungal sporocarp (10.00g). Each value is the mean of three replicate determinations ± standard deviation (n =3),[b] Total phenolic content is expressed as gallic acid equivalents (GAE; mg/g GAE). Each value is the mean ± SD of triplicate measurements.[c]Total flavonoid content is expressed as mg of quercetin equivalents (QE) per g of dry extract (d.w.)[d]Percent (%) of flavonoid content (F) in total phenol content (TP) (w/w)

Radical scavenging capacity (RSC) assay showed very high ability of extract in neutralization of DPPH· radical reaching EC_{50} value at 28.02±1.79 µg/ml (Table 2). Obtained value was ten times higher then the value for standard synthetic antioxidants (BHT, BHA), indicating lower, but close scavenging activity then pure substances. A lower absorbance at 517 nm indicates a higher RSC activity of the extract. RSC increased in dose dependent manner with the concentration showing strong activity even of 95% at 0.5 mg/ml, 5 times higher than that of other mushroom species (Barros *et al*., 2007). Comparing results for EC_{50} values of RSC activity on DPPH radicals which exhibited almost 2-3 times lower value then the Slovenian species (Mujić et al., 2010), it seems that indigenous species possess much higher RSC activity than the species from Istra and similar to *G. lucidum* methanolic extract obtained recently (Karaman, 2010).

Table 2. Antioxidant activity of examined fungal extracts, expressed as EC_{50} [µg/ml]

RSC EC_{50} [µg/ml][1]	**Antioxidative assays**	
Fungal extract	**DPPH [a] assay** (µg/ml)	**FRAP [b] assay** (mg/g)
A. aegerita	**28,88±1,39**	**13,47±2,64**
BHA[1]	2.09±0.56	
BHT[2]	8.62±0.50	
PG [3]	0.70±0.65	
r^2	0.99	

*[1]**BHA**- butylated hydroxyanisole (0.32 mol/dm3);[2]**BHT**- tert-butylated hydroxytoluene (0.25 mol/dm3);[3]**PG** - pirogalol;**r^2** - correlation coefficient between concentration and RSC at p<0.05; **RSC-** radical scavenging capacity, **EC_{50}** [µg/ml] – conc. of extracts that coused 50% of activity (neutralisation of DPPH· radicals or inhibition of cell proliferation;[b]Ferric reducing capacity of extract **(FRAP)** is expressed as ascorbic acid equivalents (AAE, mg/g d.w)*

Redox capacity of a compound may serve as a reliable indicator of its antioxidant capacity, and it is known that the effectiveness of certain antioxidants is associated with their redox capacity. Some authors considered that the redox capacity of antioxidants is generally associated with the presence of reductones, such as ascorbic acid (Duh et al., 1999). These compounds exhibit antioxidant activity in several ways, one of which is to react with certain precursors of peroxide, which prevents the formation of peroxides (Haliwell and Gutheridge, 2007).

From the results obtained in this work for *A. aegerita* (Table 2), it is evident that the mushroom extract had lower redox capacity when compared to other mushroom species (Karaman et al, 2009). It seems that this type of antioxidative mechanisms is not dedicated to the manifested activity for this species. Since phenolics are known to have ability to chelate metals, inhibit lipoxygenase and scavenge free radicals (Dubost *et al.*, 2007) we tried to detect some phenolic acids in this sample. The majority of analyzed phenolic acids were identified in *A. aegerita.* The highest content was obtained for gallic and protochatehuic acids while lower content was shown for caffeic and vanillic acid. These results support the healing properties of this edible species (Table 3). Comparing these values to the results obtained recently with other lignicolous species (Karaman et al, 2010), it seems that *A. aegerita* is

similar to *M. giganteus* species in a content of gallic acids, while having twice higher content of protochatehuic acid. It was also similar to *G. applanatum* according to the qualitative presence of all analyzed phenolic acids, containing much higher amount of all of the analyzed species except vanilic acid.

Table 3. Phenolic acid content of *A. aegerita* extract [µg/g]

Fungal species	**Phenolic acid [µg/g]**				
	Galic	Protochatehuic	Caffeic	p-cumaric	Vanilic
A. aegerita	606	696	396	nd	242

nd –not detected

Phenolic acids identified in some commercial fungi were p-hydroxybenzoic acid, p-coumaric acid, vanillic acid, tr-cinammic acid and caffeic acid (Dubost et al., 2007). Comparing these results with data obtained for *A. agrocybe* or other lignicolous fungi (Karaman et al., 2010), it can be said that the chemical composition of commercial mushroom drastically differs from the analyzed lignicolous species growing wild. Result demonstrated in this work is consistent with results obtained by comparison of commercial and wild species, according to which the amount of total phenolics was significantly higher in wild species (Barros et al., 2008). Moreover, these results could also confirm the assumption that the amount of phenolic antioxidants are dependent to a large extent on external environmental factors such as habitat, locality, or exposure to stressful influences, when the metabolic pathways of organisms are changing and moving in the direction of secondary metabolism (Puttaraju et al., 2006).

CONCLUSION

In conclusion, these results indicate that fungal species *A. aegerita* could be used as an excellent natural source of antioxidants in nutrition and as a good replacement for carcinogenic synthetic antioxidants commonly used as additives as well.
Considering the fact that ethanol extract of this species possess high yield and high RSC activity and contains phenolic acids, tinctures made of this species would be a good choice in consuming this fungi as dietary supplement in a daily nutrition, as well as in the future studies of bioactivity of this fungi.

ACKNOWLEDGMENTS

This research was supported by the Project III 46001, Ministry of Education and Science, Republic of Serbia .

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VALUE-ADDED SOYBEAN PRODUCTS AS A RESULT OF ORGANIC PRODUCTION

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ABSTRACT: According to the preliminary analysis of development indicators, food production in Serbia has been identified as one of top national priorities. The food industry represents one of the leading industries in our country, and it is developed most in Vojvodina, where it participates with 36.3% in the industrial structure (www.vojvodina.gov.rs). Serbia's food industry is comprised mostly of small and medium-sized businesses. As integral parts of all market economies, small and medium-sized businesses are important factors in economic development in all countries. These small and medium-sized businesses produce considerable amounts of food, so it is clear that food safety of their products affects the integrity of the whole food supply chain. One member of this group of businesses is the company "Galus d.o.o.", based in Pancevo, which has a business unit for the production of vegetarian food products "Soya Food", located in the village Lokve. The main activity of "Soya Food" business unit is the production and processing of soybeans and vegetarian food products. In our country, soybean is grown on an average of around 140,000 hectares, with only a slight portion being produced by organic production methods.
Starting with 2011, the company "Galus d.o.o." has introduced the organic production standard in its total production area, thereby establishing the prerequisites for the produced raw materials and subsequent finished products to carry the label "organic soybean products". This has created favourable conditions for reaping financial and ecological benefits from organic production, and added value of the existing and some new soybean products. This paper will examine in more detail the structure of this company, production and processing of organic products, and production methods regulated by law.
Key words: *value-added, soybean products, "Soya Food"*

INTRODUCTION

Small and medium enterprises (SMEs) are very important for the Serbian economy and its development, and since Serbia has become a candidate for the EU membership, European policies are increasingly affecting the operation of Serbian enterprises. Due to their large developmental importance, as well as the desire to make Serbia an equal member of the European Union - Serbia, following the EU model, is creating laws, programmes and support schemes in order to help domestic SMEs reach the European level of development and use their full development potential. In expert and political circles of the EU, SMEs have been identified as a key source of changes, competitiveness, inovation, flexibility, new jobs, and increase in productivity. They are an important factor in economic stability on both national and regional levels, the driving force behind cultural development and sustainable changes in Europe, and the engine of overall economic development. In accordance with the recognized importance, the European Union places SMEs in the center of its development policies and devises various measures and support programmes (Nikolić and Stošković, 2011).
In the Eropean Union, small and medium enterprises constitute 99% of the total number of its twenty-one million enterprises, the majority of them consisting of micro enterprises which employ 1-9 workers. SMEs are the main source of employment and wealth creation, providing for over 80 million jobs. Employees of SMEs make up two thirds of the total number of employees in the private sector, and generate 70% of the total turnover, 600,000 euros

per employee, 65,000 euros of added economic value per worker, and 60% of the EU's gross national product (Grozdanić, 2004).
In the EU these businesses are in the focus of interest of all policy and system creators who, via institutional and strategic mechanisms employed on the level of these micro economic entities, aim to create a favourable economic climate with the ultimate goal of creating an economy with the highest competitive advantage in the world. A key component of the transition process in DCs (developing countries) is the development of small and medium businesses (Babović, 2005). The development of SMEs in transitional economies is determined by initial conditions existing at the beginning of transitional process, SMEs' growth rates seen through the prism of political orientation, and potential support of the international community for the encouragement of SMEs' development.
In the upcoming period, considerable attention will be paid to “greening“, for example, crop diversification or the protection of permanent green areas. The “greening“ component in the Commission's draft, i.e. the measures defined by the term ˝greening˝, are a set of regulations which have been known for years in organic production. These regulations will come into force in 2014 as the best way to stimulate further popularization of both organic production and the practices already adopted by organic farmers. Organic production is framed by law, and compliance with these regulations is controlled according to the established procedures (organic production inspectors), evaluated and certified at the state level by issuing of the official label – “organic product“. Authorized inspection bodies (organizations, institutions) have trained and authorized inspectors for different types of production and processing.
A great demand for soybean in China has led to the increase in price of this oil crop of as much as 38% in the course of the last year. Global demand for soybeans as a favourable raw material and biofuel is on the rise. The countries which are the largest producers of soybeans are the USA, Brasil and Argentina, and they produce GMO soybeans.
For this reason, the demand of foreign buyers and primarily buyers from the EU for domestic organic soybeans is growing significantly every year.

Case study – a small enterprise “GALUS“ doo Pancevo, business unit RJ “SOYA FOOD“ Lokve in organic production

SMEs are usually defined by using various quantitative parameters such as: the number of employees, investment scope, the size of available capacities, etc. In the EU legislature, “small enterprise“ is a term which refers to companies with 50 to 100 employees (Radenković-Jocić, 2002). In our country small and medium enterprises are not defined by law, but the term “small enterprise“ usually refers to companies with under 50 employees (Bešić, 2009). One such company is Pancevo based “GALUS“ doo, which was founded in 1992. This enterprise has 12 employeees, one with a university diploma (education level VII in Serbia) and 11 employees with high-school diplomas (level IV). The company's activity is “Manufacturing of other foods“, and in 2005 this firm opened ˝Soya food˝ plant for the production of vegetarian food in order to expand its business.
“Soya Food“ plant for the production of vegetarian food is located in the village Lokve (San Mihai in Romanian) in Southeast Banat, 60 km from Belgrade and 40 km from Vrsac, in the Municipality of Alibunar. “Soya Food“ is a relatively new company founded by Ilija Stanču and his family in Lokve (soya-food.net, 2012). The production plant and storage area cover 260 m^2. This company submitted the application for the HACCP certification in 2011, thereby gaining competence in addressing HACCP principles which apply to the needs of small enterprises, which have been introduced by the certification body “AQA“ d.o.o. from Belgrade. In the upcoming period, the company's management plans to introduce the ISO 22000:2005 standard, the role of which is to provide food safety management.
Since 2008, the company has been cooperating with ˝Tamiš Institute“. In 2011, the company adopted a new approach to its future policy and development by introducing organic production over its complete area of 36 hectares, under the control of the certification organization ˝Ecocert Balkan˝ from Belgrade.

“Soya food” products

In the Scientific and Technological Development Strategy of the Republic of Serbia 2009-2014, innovation in food production is the area of research with top-priority. In addition, the new EU Common Agricultural Policy Draft for the period from 2014 to 2020 brings to the fore research and innovations, the production of ecological products, ensuring food safety and opening new jobs. Innovations, organic farming and the young, as the three cornerstones of the future EU Common Agricultural Policy will also be the field of activity of Serbian agriculture which has recognized organic production as one of the top priorities in the future strategy for agriculture and rural development (Filipović i sar., 2012).
Innovativeness in the work of ˝Soya food˝ team was evident in the preceding period as well, when it cooperated closely with a number of research organizations, one of which being the Faculty of Agriculture in Novi Sad. The company's product range currently consists of 18 different products, which will in the near future bear the prefix “organic“ and be labelled with organic logo. All of these products are being sold in domestic market and in Bosnia and Herzegovina. “Safe food's“ product range includes: three types of soy milk (natural, cinnamon and vanilla), six kinds of tofu (natural, with cumin, with dill, with paprika, with sesame seeds, and “Dimsi“ silken tofu), two kinds of tofu cream cheese (with horseradish and with dill), two types of vegetarian pates (vegetarian and vegetarian special), four kinds of vegetarian sausages (frankfurter vegetariana, sausage vegetariana, vegetarian roast with paprika – salami and vegetarian bologna), and one kind of mayonnaise (low-calorie). All products can be classified as safe and functional foods, containing no artificial preservatives, artificial dyes, nor arficial aromas.
In a subsequent part of this paper we will examine only one soybean product – soy milk. Soy milk is known to be rich in vitamins and minerals, it doesn't contain lactose or cholesterol, and its saturated fat content is very low. Apart from other vitamins, it is particularly rich in the B-group vitamins and vitamin E, and contains minerals such as phosphorus, magnesium, and iron. It has a delicious taste and refreshing aroma, and it can be used in the preparation of sweet puddings, muesli, sauces, milk shakes, nescafé, etc. It is lactose-free and gluten-free. It is not recommended as a substitute for cow's milk for children under 12 months. Technological process of soy milk production is represented in Figure 1.

Organic production - ˝Soya food˝ Lokve

Our laws and subordinate legislation are to a large extent in compliance with European legislation. They protect organic producers from various forms of fraud and incorrect and false representation in the market, enabling control over manufacturing, storing, transporting and marketing (Filipović, 2005). There is a great interest in marketing our “organic“ products to the European market, however, a lack of activity on our part has opened up this window of opportunity to the countries which, despite their poorer product quality, narrow product range and exessive distance, have managed to break into this picky market (Filipović, 2008).

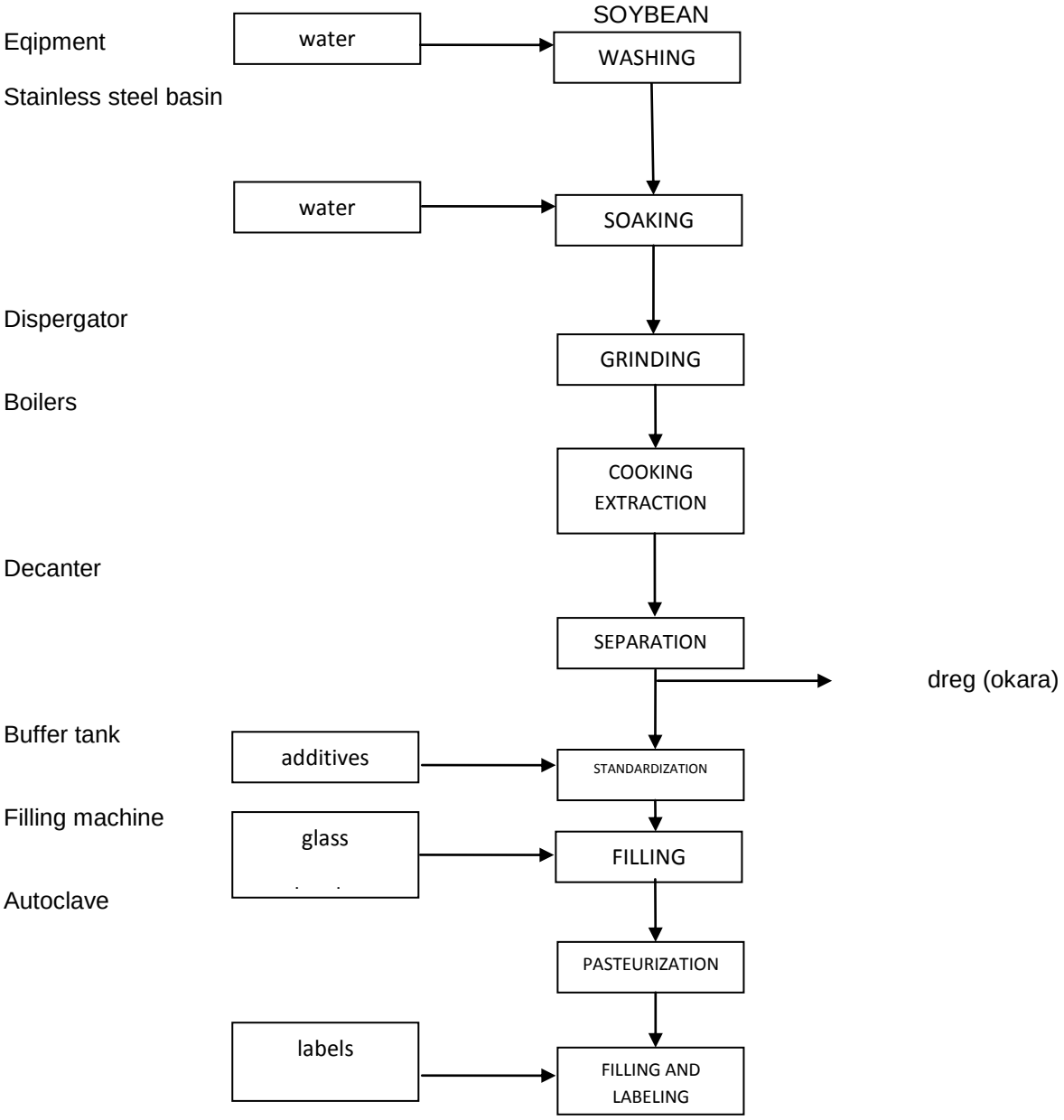

Figure 1. Flowchart representing soy milk production (Popović-Vranješ et al., 2007)

Sustainable development and economical and ecological profits generated in organic production provide opportunity for the improvement of the existing production. In organic production (and processing), standard agronomic knowledge is being built into regulated methods of production, packaging, storing and transport. Organic compliance is controlled by means of established procedures (organic production inspectors), evaluated and certified at the state level by issuing of the official label – “organic product“. All segments of production, control and certification are covered by the law and rulebooks (Organic Law, 2010; Organic rulebook, 2011).

SWOT analysis of organic production in RJ ˝Soya food˝ Lokve

SWOT analysis can serve as an appropriate tool which helps a company develop its business in conditions where there are external opportunities and threats, and internal strengths and weaknesses. SWOT analysis of this company's present and future opportunities, external threats, and internal weaknesses was performed from April 2011 to February 2012. Mr Ilija Stanču, the owner of “Galus”, and Mr George Munćan, ing., the chief technologist in RJ ˝Soya food˝ have given their full support during the analysis. The analysis was conducted in the following stages: 1. Performing of internal analyses [main internal strengths and critical internal weaknesses], 2. Performing of external analyses [opportunities in the environment and main threats in the environment]. After comparing external (threats and opportunities) and internal (strengths and weaknesses) variables, four types of strategy can be identified:

1. Mini-mini strategy (WT) which attempts to find ways to minimize internal weaknesses and avoid external threats.
2. Mini-maxi strategy (WO) which attempts to find ways to minimize internal weaknesses and maximize external opportunities.
3. Maxi-mini strategy (ST) which attempts to find ways to minimize external threats and maximize its strengths.
4. Maxi-maxi strategy (SO) which attempts to find ways to take the maximum advantage of its external opportunities by the maximization of its internal strengths.

Table 1. SWOT analysis – organic production in the case study - RJ ˝SOYA FOOD˝ Lokve

Strengths	**Weaknesses**
- Nearness to raw materials - Tradition in and knowledge of conventional agricultural production of vegetarian food - Membership in associations whose principal activity is organic production - Favourable location of the enterprise - High level of technological competence - Concept of cooperation with scientific research institutions - Existence of HACCP system	- Lack of basic raw materials for the production of some products - Lack of knowledge about foreign organic production regulations - Low interest of local community in organic production - Lack of active cooperation between organic and conventional producers - Nonexistence of a transparent data base of organic food producers and retailers
Opportunities	**Threats**
- Export potential for marketing soybean products - Association within a sector and between sectors (clustering) - Harmonisation of legislature with the EU and international standards - Potential cooperation and partnerships among a large number of "young" producers - Expected growth of organic food consumption per capita - Products protected by a recognizable brand name ˝Soya food organic˝ - Export potential - Widening of the current product range - Easier integration into the EU via organic production - Purely competitive market	- Insufficient political awareness of the vital role of organic farming in the restructuring of agricultural sector in the process of accession to the EU - Marginalization of organic production by various economic entities - Potential negative impact of current bureaucracy on all production stages, from the purchase of repro materials, through incentivising, to export - Foreign threats and the need to 'protect' domestic organic food from the imported - Non-compliance with international organic production regulations - Low purchasing power of the domestic market - Political instability

CONCLUSION

From everything mentioned above it is clearly necessary to encourage the development of small enterprises in the domestic food industry, while at the same time taking care of the public health protection. It is therefore important to devise a strategy for food safety and integration of HACCP standards into SMEs. Although they are not primarily food exporters, SMEs have a large influence on national and regional economies and a potentially strong influence on the health of domestic consumers, and consequently on overall national health. The strategy for further development of "GALUS" doo company from Pancevo, i.e. its branch, RJ ˝SOYA FOOD˝ Lokve, is ongoing work and improvement with the goal of producing safe food. The company's determination to certify its whole production and processing as organic is in alignment with the policies of the EU and our country. Unfortunately, there are some obstacles on this road, which have been presented in this paper through the SWOT analysis.

ACKNOWLEDGEMENTS

This paper is a represents a part of the research results within the Project of Integral and Interdisciplinary Research No. 046006 "Sustainable Agriculture and Rural Development in the Function of the Accomplishment of Strategic Objectives of the Republic of Serbia in the Danube Region", financed by the Ministry of Education and Science of the Republic of Serbia.

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BEER AS AN INTEGRAL PART OF HEALTHY DIETS – CURRENT KNOWLEDGE AND PERSPECTIVE

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ABSTRACT: Beer is one of the oldest beverages. Throughout history, it has been accepted not only as a refreshing drink, but also as a liquid bread, a source of energy, a healing remedy and, lastly, as a safe drink that can promote well being. Nowadays, beer is one of the most popular beverages all over the world and an integral part of diets in most countries. For the last decade, it has been in focus of significant medical research and the numerous results have given the scientific confirmation of the statement known for centuries: beer is far more than a thirst-quenching low-alcohol beverage. Published data have indicated that beer contains a wide range of nutrients with bioactive properties and, if consumed moderately and in a responsible manner, can be a useful part of a healthy diet. Of particular interest are its polyphenol, mineral, trace element and vitamin composition, as well as its antioxidant properties. It seems that we have reliable enough results to claim that beer has a potentially positive influence on low blood pressure, atherosclerosis and cardiovascular diseases, and may help protect against gallstones and kidney stone formation, dementia, osteoporosis etc. However, several facts deserve to be discussed: the latest information considering the beer's beneficial action, the meaning of moderation in each particular case, the significance of beer versus wine in health promotion, the thirst-quenching effect as a low-alcohol beverage, the influence on the energy intake and the problem with obesity, and, finally, the perspective in terms of the new beer types with new sensory and functional properties.
Key words: *beer, bioactivity, antioxidants, minerals, cardiovascular diseases*

INTRODUCTION

Beer has been drunk for more than 8000 years, from the time when it was first made probably unintentionally during mid-ancient times. For centuries, it has played an important part in many cultures, serving as a warming beverage, and safe drink in an age when the purity of water was uncertain. Ever since, it has been accepted not only as a refreshing drink, but also as a liquid bread, a source of energy, and, a drink that can promote well being (Bamforth, 2000). For the last decade, it has been the focus of significant medical research and the numerous results have given the scientific confirmation that beer is far more than a thirst-quenching low-alcohol beverage. Published data have indicated that beer contains a wide range of nutrients with bioactive properties and, if consumed moderately it can be a useful part of a healthy diet, improve well-being, and reduce risk of various types of diseases.

BIOACTIVE COMPONENT IN BEER

Beer is an extremely complex beverage. Apart from water, which normally represents more than 90%, and about 5% v/v of ethanol, beer contains about 800 organic compounds and many of them are biologically active. Potentially beneficial effects on the human body are the consequents of two facts, the small amount of alcohol and the presence of other compounds, such as vitamins, minerals, trace elements, phytoestrogens, and antioxidants. Numerous studies confirmed that beer consumption in moderation could provide a considerable increase of B vitamins in human body (Table 1) (Bamforth, 2002). From the nutritional point of view and the position of beer as a part of the diet, the most important minerals are potassium, magnesium, sodium, and phosphorus. Beer is rich in potassium and magnesium, low in sodium and calcium,

and may be a significant dietary source of phosphorus, and even selenium, and silicon (Table 2) (Buiatti, 2009; Leskošek-Čukalović, 2009).

Table 1. The composition of beer relative to recommended dietary intakes of vitamins

Parameter	*Daily adult (age 25 – 50) requirement*		*Range in beer (per litre)*
	Male	*Female*	
Energy (Kcal)	2550	1940	150-1100
Protein (g)	63	50	3-5
Vitamin C (mg)	60	60	0 -30
Thiamine (mg)	1.5	1.1	0.003 – 0.08
Riboflavine (mg)	1.7	1.3	0.02 – 0.8
Niacin (mg)	19	15	3 - 8
Vitamin B_6	2.0	1.6	0.07- 1.7
Folate (µg)	200	180	40 - 600
Vitamine B12 (µg)	2	2	3 - 30
Biotin (µg)	30 - 100	20 - 100	2 - 15

Table 2. Mineral content of beer

Mineral	*Beer (mg/L)*	*Mineral*	*Beer (mg/L)*
Potassium	200 – 600	Silica	40 - 120
Sodium	10 - 100	Phosphate	260 - 995
Magnesium	60 - 250	Sulphate	60 - 300
Calcium	20 - 160	Chloride	150 - 400
Iron	0.01 – 0.3	Selenium	<0.0004 – 0.0072
Copper	0.02 – 0.4	Lead	< 0.01 – 0.1
Zink	0.02 – 4.5	Fluoride	0.09 – 0.2
Manganese	0.03 – 0.2	Cobalt	0.01 – 0.11

Polyphenols from malt and hops are the major natural antioxidants in beer. There are many references in literature determining their content of beer. Identified are 78 different phenolics compounds including simple phenolics, aromatic carboxylic and phenol carboxylic acids (Gerhäuser, 2005; Gorjanović et al., 2010). The antioxidant capacity of beer depends a lot on beer type. Dark beers seem to be considerably inferior to coffee, red wine and tea, but can be compared to rose and white wine, and orange juice (Table 3) (Lugasi and Hóvári, 2003).

Table 3. Antioxidant capacity of beverages (FRAP and ABTS assays)

Beverage	FRAP (µmol Trolox/100 ml)	ABTS (µmol Trolox/100 ml)
Coffe	2,267 ± 18.9	1,328 ± 5.1
Tea	601 ± 5.5	631 ± 8.0
Red wine	1,214 ± 24.5	1,093 ± 54.2
Rose wine	286 ± 39.2	261 ± 23.7
White wine	154 ± 36.8	181 ± 22.2
Orange juice	515 ± 41.5	249 ± 3.4
Cola	20.7 ± 0.7	≤10
Lager beer	139.6-149.5	220.0-305.6
Dark beer	278.8	259.0-536.5
Alcohol-free beer	75.6-91.2	155.8-175.3

Prenylflavonoids in beer that deserve special attention are xanthohumol (XH), isoxanthohumol (IX) and 8-prenylnaringenin (8-PN). They are present almost exclusively in hops and beer is practically the only foodstuff in which they can be found. Their final content in beer strongly depends on the production process. Although XH is the main prenylflavonoids in the hop cone, their abundance in beer is much lower. During the wort boiling much of the XH is converted by thermal isomerization into IX therefore, IX and 8-PN are the main prenylflavonoids in beers (Table 4) (Possemiers et al., 2009).

Table 4. Prenylflavonoid contents in beer and dietary supplements

Beer (µg/l)	*xanthohumol*	*isoxanthohumol*	*8-prenylnaringenin*	*Total*
Pils	9-34	400-1060	13-21	460-1100
Lager	0.2-28	20-1910	2-175	24-2342
Porter	690	1 330	240	2900
Stout	340	2100	69	2680
Hefeweizen	5	300	8	330
Strong ale	240	3440	110	4000

BEER IN RELATION TO HEALTH

During the past decades, numerous prospective population studies have described the impact of alcohol intake on all-cause mortality as a J-shaped curve, indicating that there is a beneficial effect of light to moderate alcohol intake and a detrimental effect of high alcohol intake. It has been evident that heavy uncontrolled drinking carried major medical risks, and is often considered as one of the leading causes of preventable deaths. The main causes claim to be coronary heart disease (CHD) and other athero-thrombotic diseases. However, there is also substantial evidence now that the intake of light to moderate amounts of alcohol beverages is associated with reduced morbidity and mortality from CHDs. The main beer constituents responsible for beneficial action on the diseases of vasculature system are alcohol, polyphenols, well balanced array of minerals (most important are phosphoric acid, potassium, magnesium and sodium) and vitamins (folate, B12 and B6). Phosphoric acid and magnesium are known to be important for the proper blood vessels functioning, while the high potassium and low sodium content is the right balance for healthy, low blood pressure. Moderate beer drinkers usually have a lower blood pressure than abstainers and heavy drinkers. This effect on blood pressure cannot just be explained by a vasodilatation action of alcohol, because changes also occur in the hormone, water and electrolyte balance (Winkler et al., 2006). Folate and vitamin B12 and B6 are associated with homocysteine as an independent risk factor of cardiovascular disease and its involvement in mechanisms of thrombosis (Tamai et al., 2011).

Beer is intensively analyzed in connection with other diseases as well: carcinogenesis, osteoporosis, kidney stones, dementia, etc.

Concerning malignancy there is convincing evidence that high alcohol intake is related to carcinogenesis. The available evidence indicates that the alcoholic beverage consumption considered to be causally related to cancers of the upper aerodigestive tract (oral cancer and cancers of the oropharynx, hypopharynx, larynx and oesophagus), liver, and probable to colorectal cancer in men, and breast cancer in women (Gerhäuser,2005). Beer drinking is almost invariably related to alcohol consumption. However, in 1996 beer was first reported to possess antimutagenic components (Arimoto-Kobayashi et al., 1999).

Scientific evidence accumulated over the past decade points to the cancer preventing potential of XH and hop bitter acids. The latest studies have proven that XH shows cancer chemopreventive activities, antimutagenic and anticarcinogenic properties with an exceptional broad spectrum of inhibitory mechanisms at all three stages of the carcinogenesis, initiation, promotion, and progression (Gerhäuser, 2005; Ferk et al., 2010). 8-PN is of special interest as the most powerful phyto-oestrogen found in nature (Chadwick et al., 2006) and might exhibit several biological activities (Strathmann et al., 2010). Until recently, possible health effects after moderate beer consumption related to the presence of 8-PN were considered negligible because concentrations in beers are generally very low. The latest study shows that this approach may no longer be valid. Data show that moderate beer consumption can lead to 8-PN exposure values of 1-2 mg/d that might fall within the range of human biological activity (Possemiers et al., 2006; Arimoto-Kobayashi, 2005).

The available knowledge on the relationship between the consumption of alcoholic beverages and a variety of human cancers is based primarily on epidemiological evidence. For moderate consumption (2-3 drinks/day for men and 1-2 drinks/day for women), the risks never increase above twofold and are mostly less than 25% above baseline. The evidence from the epide-

miological literature suggests that 25g/d of alcohol is associated with a relative risk of 1.9 for cancers of the oral cavity and pharynx, 1.4 for cancers of the esophagus and larynx, about 10% for colorectal cancer (mechanisms unclear), and 20% for liver cancer (mechanisms well described). The association between alcohol and breast cancer is not strong and not necessarily causative, at least for moderate consumption (WHO/IARC, 2010).
Silicon impacts bone mineral density in humans and supplementing in the diets of women with osteoporosis increased bone mineral density. Silicon increases type I collagen synthesis and promotes the differentiation of osteoblast-like cells (Jugdaohsingh et al., 2004). Silicon is present in beer in the soluble form of orthosilicic acid which is >50% bioavailabile. Therefore, beer has been claimed to be a major contributor to the overall silicon intake in the Western diet (44% in adult males) (Troy et al., 2010).
The prevalence appears kidney stones have increased in the last quarter of the 20th century for men and women. Nowadays, numerous observational and experimental data obtained confirmed that daily consumption of beer (330ml, 4.5%w/w) may reduces the risk of developing kidney stones by 21-40% (Curhan et al., 1996; 1998). The protective effect of beer could be mediated through water, alcohol and mineral composition, high potassium and magnesium content, and diuretic properties.
Dementia as a serious loss of global cognitive ability is not a single disease, but rather set of signs and symptoms in which affected areas of cognition may be memory, attention, language, and problem solving. Although the adverse effects of excessive alcohol intake are well known results of many studies suggest that in older people, small to moderate amounts of alcohol consumption (beer, wine, or spirit) are associated with reduced incidence of dementia and Alzheimer's disease (Stampfer, 2005). Several mechanisms have been proposed to explain the association of moderate alcohol consumption with better cognition. In the case of beer, very high and significant correlations were found suggesting that moderate beer consumption, due to its alcohol and bioavailable silicon content, possibly affording a protective factor for preventing Alzheimer's disease. Aluminum is a highly neurotoxic element that may be involved in neuronal degeneration in human brains. Silicon and silicic acid may decrease aluminum bioavailability by blocking its uptake through the gastrointestinal tract and by impeding reabsorption (González-Muñoz et al., 2008).

INFLUENCE OF BEER ON THE ENERGY INTAKE

The position of alcohol beverage as part of the diet is defined with their alcohol content and its contribution in energy intake. The influence of alcohol on food consumption and availability is diverse. Alcohol can alter appetite for food modifying hormonal processes that have been implicated in appetite regulation. Moreover, it has influence on metabolic parameters related to obesity and has other effects on many neurochemical systems (Monteiro et al., 2009).
Alcohol is an energy-providing item, with an energy content of 29 kJ/g (7.1 kcal/g). It has no storage capacity, but its consumption can lead to positive fat balance through the sparing effect on fat oxidation, leading to increased fat storage (Yeomans, 2004). Few studies have reported a J-shaped relationship between alcohol consumption and body mass index and waist-to-hip ratio such that light to moderate drinking has beneficial effect in reducing weight whereas non-drinking and heavy or risky drinking having the opposite effect (Lukasiewicz et al., 2005).
Beer as an alcohol beverage is rather specific. Besides alcohol, it contains carbohydrates (more than most other alcohol beverage), and possesses defined calorific value. For a long time it has been blamed as the cause of overweight, waist circumference, particularly in men – a phenomenon popularly referred to as "beer belly".
Beer caloric content depends on the beer type (amount of alcohol and extract contained). For example, beer with a 3.2%w/v extract, 5%v/v alcohol content contains approximately 42 kcal per 100 ml. Assuming that the daily caloric requirements of an adult averages to 2,500 kcal, the caloric content of 1 l of beer represents approximately one-sixth of the daily requirement.
On the other hand, the residual starch-derived carbohydrate in beer (isomaltose and other dextrins) are classified as the less digestible, "slow release" carbohydrate polymers, namely "good carbs". Furthermore, one of the major carbohydrate fraction present in beer are the β-

linked materials derived from the β-glucan and arabinoxylan present in barley. Such molecules are metabolized neither by brewing yeast nor by the human body and they are highly likely to be of value as pre-biotics (Bamforth, 2007).

BEER *versus* WINE IN HEALTH PROMOTION

Now there is a growing body of evidence from studies performed in humans that supports a connection between regular moderate wine drinking and improved health. Red wine is almost overall accepted as unique drink among alcoholic beverages with most efficient health promotion action (phenomenon known as French paradox). Numerous studies that have been carried out in the health-wine field, affirmed that red wine, thanks to its unique polyphenols content and composition, supplementing the regular diet increases the total antioxidant capacity in plasma, HDL lipoprotein, fibrinolytic and antithrombin activity, reduces oxidative damage and platelet aggregation etc. (Yoo et al., 2010; Fernández-Mar et al., 2012). The mechanisms responsible for the health effects of wine are extremely complex. Both the alcohol and the polyphenol components have been extensively studied and there is controversy over which component is more important (Hansen et al., 2005). Some controversy still exists over whether red wine has superior protective effects than other alcoholic beverages as well (Guilford and Pezzuto, 2011). Several review articles have discussed the relation between the types of alcoholic beverage consumed (wine, beer, liquor) and the risk of CVD and they did not find that one type of drink was more cardioprotective than the others (de Lorimier, 2000).
Numerous studies have investigated whether the risk for different kind of cancer depends on the type of alcoholic beverage consumed as well. The results obtained are sometimes contradictory and again misleading. In other words, the strength of the apparent protective effect of different beverages is still disputed (Fillmore et al., 2006; Grønbæk, 2009).
So, what is going on? Is red wine really better than beer in health promotion? Perhaps it is, but few facts deserve to be mentioned.
Antioxidant properties (AO) of both wine and beer are well investigated (Ghiselli et al., 2000; Rivero et al., 2005; Gorjanović et al., 2010). Recent studies suggest that amount of phenolics in beer and wine might be comparable. Results obtained *in vivo* confirm that AO capacity of beer and red wine are similar despite different concentrations of total polyphenols, possibly due to superior absorption of the beer phenolics as compared with those in red wine (Gorinstein et al., 2000; Nardini et al., 2006). Although beer per drink (of equivalent alcohol content) contains more than twice as many of the AOs as white wine and only half the amount in red wine, the red wine AOs may be larger molecules that are not as readily absorbed as the smaller AOs in beer (Ghiselli et al., 2000).

THE THIRST-QUENCHING EFFECT AND REHYDRATION

Beer can be regarded as a valuable rehydration beverage particular after hard physical working and sport training and exercise. Beer is truly a natural drink, more than 90% of water and is a thirst quenching drink, which contains relatively low content of alcohol, contains electrolytes and well digestible carbohydrates for efficient energy recovery. The osmotic pressure of beer is on average 1012 mmol/kg, while in the case of alcohol-free beer it is value of 290 mmol/kg, the same as blood (Mettler et al., 2006). Alcohol-free beer can thus be classified as an isotonic drink and be regarded as a most natural drink for thirst quenching. The sugars, salts, and bubbles of carbon dioxide stimulate fluid absorption and promote rehydrating effect so that alcohol diuretic effect is minimized significantly (Walzl, 2005).
However, beer is an alcohol beverage, and alcohol deserves special attention regarding well-known ethanol diuretic effect. However, results suggest that this effect is negligible when alcohol is consumed in dilute solution as beer is and after a moderate level of hypohydration. There appears to be no difference in recovery from dehydration whether the rehydration beverage is alcohol-free or contains up to 2% alcohol. Drinks containing 4% alcohol tend to delay the recovery process (Maughan, 2000). In other word, alcohol concentrations like

those in beer may not adversely affect long-term rehydration, but should not be relied upon when rapid and complete rehydration is desired. The greatest complaint regarding beer as rehydrating drink is its low potassium content.

NEW BEER TYPES WITH FUNCTIONAL PROPERTIES

According to market statistics, consumers are increasingly interested in the health benefits of foods. This interest combined with a more widespread understanding of how diet affects disease, rising health-care costs and an aging population are driving a growing market for products with functional properties. Beer as a natural drink, which already possesses a lot of functional properties can be an excellent base for developing new products with additional benefits and functionality. Several products like this have been already present on the market.

In 2004, two new beverages were introduced in the German market. Weihenstephan brewery launched, XAN Hefeweissbeer (XAN wheat beer) with elevated levels of XN up to 1.4 mg/L, and XAN Wellness (beermix with 40% of alcohol-free wheat beer and 60% natural multi-fruit juice -apple, acerola, lemon- and, advertised as the first ever soft drink to contain XN) with 4 mg/L of XN. Soon after Žatecký brewery in Czech Republic promoted ZATEC Xantho, dark beer with 0.3 mg/L of XN and 3 mg/L of IXN.

German brewer Karlsberg (not to be confused with Danish Carlsberg) has launched functional beer for women Karla - the mixed drink in two varieties. Both were low in alcohol content (1%v/v) and a blend of beer and fruit juices. *Karla Balance* claimed to provide 'peace and balance' by mixing hops with lemon balm, a herb well-known for its sedative properties. *Karla Well-Be* was enriched with ingredients such as soy-derived lecithin (which may positively affect cholesterol levels), folic acid (recommended for women considering pregnancy), and other vitamins. Emphasis on health, Karlsberg supplied its beer through an unusual distribution channel: pharmacists. However, although singled out by Polish business magazine Handel as Poland's best new FMCG product of 2006, Karla cannot be found any more on Karlsberg's web site (www.karlsberg.de).

There are also several beers with added functional additives or modified content to satisfy defined target group of costumers. One example is *Spirulina beer*. Spirulina is blue-green algae that contains numerous bioactive components and can be consumed as a dietary supplement, as a functional food additive, as well as a whole food. It is supposed to promote health in many ways: protects from oxidant stress and supports the immune system and a healthy inflammatory response. It is cultivated worldwide and is available in tablet, flake and powder form, now as a beer as well. Spirulina beer is claimed to keep the nutritional components and flavor of traditional beer, and increases its nutrient content derived from Spirulina (Liang et al., 2004). Gluten-free beer is another example. A celiac disease or gluten intolerance is a digestive disease that damages the small intestine and interferes with absorption of nutrients from food. People who suffer from this disease cannot tolerate gluten, a protein in wheat, rye, barley, and food containing these cereals, such is beer. Nowadays gluten-free beer is a solution for all those who suffer from such a disease and cannot allowed themselves regular beer. These beers are produced mostly with buckwheat and sorghum as the substitutes for barley molt (Bamforth, 2009).

A variety of plants, medicinal herbs, and mushrooms give a lot of possibilities to create a new beer line with defined functional properties and therapeutic action as well. During the past decades, public interest in natural therapies, namely herbal medicine, has increased dramatically. This fact may be the challenge for brewing industry to make a breakthrough for the growing functional food market. Possibilities are enormously high (Despotović et al., 2007; Leskošek-Čukalović et al., 2010a; Leskošek-Čukalović et al., 2010b; Veljović et al., 2010).

CONCLUSION

Studies focusing on the associations between different health conditions and alcohol consumption confirm the hazards of excess drinking but also indicate the existence of potential windows of alcohol intake that may confer a net beneficial effect of drinking, at least in terms of survival, both in men and in women. However, available epidemiological studies in many cases have provided contradictory results, showing positive, or negative, or non-significant associations between alcohol consumption and prevalence for given diseases, differences between type of alcohol beverage, active constituents and mechanisms of their beneficial action. In many cases, they have yielded no conclusive evidence that alcohol either promotes or prevents diseases. Therefore, the question whether to drink or not is still open. Moderate drinking seems to be beneficial in prevalence of some diseases but to recommend anyone to drink alcohol because of that would be very dangerous. Beer is a drink with numerous constituents important for healthy body functioning and regarding alcohol content is less hazardous than other alcohol beverages, but not an exception in terms of binge drinking. All studies found a negative association between alcohol consumption and health, especially in heavy drinkers and binge drinkers, regardless the type of alcohol.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III046001 financed by the Ministry of Education and Science, Republic of Serbia.

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ANTIOXIDANT PROPERTIES OF GENISTEIN AND DAIDZEIN – TWO POTENT SOYBEAN ISOFLAVONES

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ABSTRACT: Soybean and full-fat soybean products contain approximately 20% of oil and are susceptible to lipid peroxidation, particularly during the storage and distribution of raw material and thermal treatments used in full-fat soybean production.
Antioxidant properties of soybean isoflavones (genistein and daidzein) have been investigated in soybean oil model systems by measuring the ability of genistein and daidzein to inhibit the formation of radicals which were stabilized in the presence of spin trap N-*tert*-butyl-α-phenylnitrone (PBN) and detected in by electron spin resonance (ESR) spectrometry.
In both thermal and catalytic oxidation of soybean oil, the same hyperfine coupling parameters (a_N=14.75 G and a_H^{β}=2.80 G) confirmed the generation of PBN-OOL/-OL spin adducts (lipid oxyradicals). The antiradical activity of genistein and daidzein, in the 0.005-0.02% concentration range, was tested by measuring their ability to inhibit the formation of lipid oxyradicals during thermal and catalytic oxidation of soybean oil.
The antioxidative nature of genistein and daidzein was also tested by using DPPH test and chelating activity on Fe^{2+} assay.
It has been found that genistein inhibits the thermal oxidation of soybean oil by decreasing the concentration of lipid oxyradicals, while during the inhibition of catalytic oxidation of soybean oil it acts as a chain-breaking antioxidant and a metal chelator. Daidzein acts as a very weak chain-breaking antioxidant which does not demonstrate chelating activity on Fe^{2+} ions.
Key words: *genistein, daidzein, lipidoxyradicals, ESR, DPPH radicals, chelating activity*

INTRODUCTION

Soybean and full-fat soybean products contain approximately 20% of oil and, therefore, they aresusceptible to one of the non-desirable reactions of foods – lipid peroxidation. Thermal treatments which are used during the production of some soybean based foods could cause thermal oxidative degradation of soybean lipids. Also, soybean contains catalytically active metal ions which could act in catalytic oxidative degradation of soybean lipids. Full-fat soybean products could also be prone to lipid peroxidation during storage, as well as during distribution of foods, when susceptibility increases due to exposure to light (photooxidation) or air (high contact surface) (Namiki, 1990).
As lipid peroxidation represents one of the greatest problems during the production, storage and distribution of foods and feeds, it is necessary to reduce or eliminate the acting of the process. The use of antioxidants is one of the best ways for suppression the lipid peroxidation. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have limited use in foods, as they are suspected to be carcinogenic (Namiki, 1990). Therefore, the use of natural antioxidants or the implementation of processes that could preserve the natural antioxidant capacity of raw material during food processing has increased tremendously in recent years.

Soybean antioxidants include liposoluble antioxidants – α-tocopherol (Hamilton et al., 1997), soybean phospholipids or lecithin (Reblova et al., 1991), carotenoids (Bast et al., 1998), and non-liposoluble antioxidants, particularly soybean isoflavones (Cassidy et al., 1994; Tyug et al., 2010).
Soybean isoflavones are reported to act as phytoestrogens (Böhm and Franke, 1996), anticancerogens (Adlercreutz, 1995) and they can reduce the risk of cardiovascular diseases (Sacks et al., 2006; Tikkanen et al., 1994) and chronic renal disease (Ranich et al., 2001). The mentioned activities of soybean isoflavones are often related to their antioxitant activity (Yang et al., 2000), especially in the case of genistein (Lee et al., 2005; Record et al., 1995).
The antioxidative properties of soybean isoflavones are important for the medicinal and nutritional purposes (Mateos-Aparicio et al., 2008; Messina and Barnes, 1991; Xiao, 2008), as well as for food preservation (Pinto et al., 2005; Shimoni, 2004; Tyug et al., 2010).
There are many literature data related to the antioxidant capacity of soybean foods and feeds that is attributed to the presence of soybean isoflavones (Murphy et al., 1998; Wang et al., 1999; Hasnah et al., 2009).
Therefore, the aim of this work was to estimate the influence of genistein and daidzein on: a) thermal and catalytic oxidation of soybean oil using electron spin resonance (ESR) spectroscopy and "spin-trapping" technique b) DPPH radicals (scavenging effect) and c) concentration of Fe^{2+} (chelating effect).

MATERIAL AND METHODS

Material

Commercial soybean oil was purchased from the local market for the preparation of model systems intended for testing thermal and catalytic oxidation of soybean oil. Genistein, daidzein, N**-**tert**-**butyl-α**-**phenylnitrone (PBN), 1**,**1-diphenyl-2-picryl-hydrazyl (DPPH) and tetramethyl murexide were supplied by Sigma Chemical Company, St. Louis, MO, USA, and hexamine was obtained from Aldrich, Chemical Company, USA.

Thermal oxidation of soybean oil

The system for investigation of thermal oxidation of soybean oil (model system I) was prepared by mixing 6.0 mL of soybean oil with 21.3 mg (0.12 mmol) of spin trap N-*tert*-butyl-α-phenylnitrone (PBN) (blank probe I). Reaction mixture was heated at 60 °C. Thermal oxidative degradation of soybean oil was detected by measuring the formation of PBN lipid radical spin adducts after 24 h.

The influence of genistein and daidzein on the thermal oxidation of soybean oil was investigated by adding genistein or daidzein at 0.05-2.00% concentrations to blank probe I.

Antiradical activity (AA in %) of isoflavones was calculated according to the following equation:

$$AA=100\cdot(h_o-h_x)/h_o \qquad /1/$$

where h_o and h_x are the heights of the first peak in the ESR spectrum of PBN lipid radical spin adducts of blank probe I and probe I, respectively.

Catalytic oxidation of soybean oil

The system for investigation of catalytic oxidation of HESO (model system II) was prepared by mixing 6.0 mL of soybean oil, 3.0 mL of distilled water, 21.3 mg (0.12 mmol) of spin trap N-*tert*-butyl-α-phenylnitrone (PBN) and 3.9 mg (0.015 mmol) of ferrous sulphate heptahydrate (blank probe II). Reaction mixture was allowed to stay at room temperature (23 ± 1 °C). Catalytic oxidative degradation of soybean oil was detected by measuring the formation of PBN radical spin adducts after 3 h.

The influence of genistein and daidzein on the catalytic oxidation of soybean oil was investigated by adding genistein or daidzein at 0.05-2.00% concentrations to blank probe II.

Antiradical activity (AA in %) of genistein and daidzein was calculated according to Eq. 1, where h_o and h_x are the heights of the first peak in the ESR spectrum of PBN radical spin adducts of blank probe II and probe II, respectively.

Detection of oxygen free radicals

ESR spectra were recorded with spin trapping technique using Bruker 300E ESR spectrometer (Bruker, Rheinstetten, Germany) under the following conditions: modulation field 100 kHz, modulation amplitude 0.204 G, receiver gain 10^3, time constant 327.68 ms, conversion time 1310.72 ms, centre field 3440.00 G, sweep width 100.00 G, microwave frequency 9.64 GHz, microwave power 20.0 mW, and temperature 23 ± 1 °C.

Magnetic field scanning was calibrated using Fremy's salt (peroxylamine disulphonate). A quartz flat cell Bruker ER-160FC was used for detection. Splitting constants were calculated from computer-generated second derivatives of the spectra after optimizing signal-to-noise ratios and were verified by computer simulations. ESR spectral files were imported into the WinSim program (WinSim, Sugar Land, TX, USA) for the analysis of the hyperfine splitting constants (Duling, 1994).

Scavenging activity on DPPH radical

Different concentrations of genistein and daidzein solutions (0.25 mM, 0.50 mM, 1 mM, 2 mM) (80% ethanol, 4 mL) were mixed with a methanolic solution of DPPH radicals (1 mL), and the final concentration was 0.2 mM. The absorbance was measured after 30 minutes at 517 nm (Shimada et al., 1992).

Chelating activity on Fe^{2+}

Chelating effect was determined following the method of Shimada et al. (1992). Different concentrations of genistein and daidzein solutions (0.25 mM, 0.50 mM, 1 mM, 2 mM) (80% ethanol, 2 mL) and tetramethyl murexide (1 mM, 0.2 mL) were added into the mixture (2 mL) of 30 mM hexamine, 30 mM KCl and 9 mM ferrous sulphate ($FeSO_4$ x $7H_2O$). The absorbance was measured after 3 minutes at room temperature (23 ± 1 °C) at 485 nm.

RESULTS AND DISCUSSION

The thermal and catalytic oxidation of soybean oil in the investigated systems (model systems I and II) was registered by recording ESR spectra of PBN spin adducts of formed radicals during the reaction period of 24 h at the thermal oxidation and during 3 h of the catalytic oxidation of soybean oil.

The ESR spectra recorded in both model systems consist of six lines of the same relative intensities, characteristic for the interaction of unpaired electron and ^{14}N-atom (I=1) and ^{1}H-atom (I=1/2), with hyperfine coupling parameters (a_N=14.75 G and a_H^{β}=2.80 G). Radicals trapped in the investigated systems are recognized as lipid oxyradicals, i.e. spin adducts obtained in these systems are marked as PBN-OOL/-OL spin adducts (Sakač et al., 2010).

PBN-OOL/-OL spin adducts were also detected in model systems I and II in which genistein or daidzein was added, but their formation was affected by the addition of genistein in both model systems in the investigated concentration range, while daidzein did not manifest antioxidant activity in model system I, but slightly suppressed the formation of PBN-OOL/-OL spin adducts in model system II (Fig. 1).

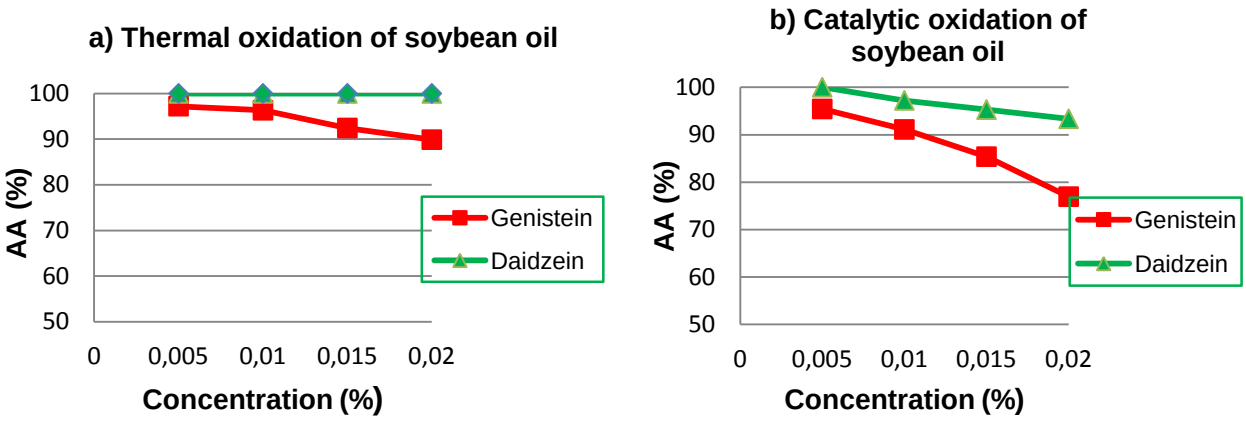

Fig.1. Antioxidative activity of genistein and daidzein on PBN-OOL/-OL spin adducts formed during thermal (a) and catalytic (b) oxidation of soybean oil in model systems I and II

The obtained results (Fig. 1) suggest that genistein acted as a relatively weak chain-breaking antioxidant in the model system I, capable to scavenge free radicals and thus preventing the thermal oxidation of soybean oil. In contrast, it acted in two ways in model system II – as a chain-breaking antioxidant and as a metal-chelator.

The statement that genistein, but also daidzein, although weakly, could act as an antioxidant by donating H-atom was confirmed in DPPH assay (Fig. 2) in which a dose-dependent scavenging activity was observed for both isoflavones.

Chelating properties of genistein were evidenced during measurement of chelating activity on Fe^{2+}, while daidzein expressed extremely weak tendency to chelate Fe^{2+} (Fig. 3).

The antioxidant effectiveness of genistein as a chain-breaking antioxidant, which donates H-atom to DPPH radicals (Fig. 2), was indicated by Record et al. (1995). Yang et al. (2000) reported that the fermented soy drinks, known for their high content of isoflavone aglycones, showed a relatively strong scavenging activity on DPPH radicals. The scavenging activity of genistein and daidzein was also recorded by Mitchell et al. (1998), who used ESR spectroscopy to monitor the scavenging activity of a number of phytoestrogens in the presence of galvinoxyl-radicals. Their results demonstrated that genistein and daidzein did not strongly scavenge DPPH or galvanoxyl radicals. Lee at al. (2005) also found out that genistein and daidzein possess antioxidative capacity, but they were less effective compared with known potent antioxidants, such as epicatechins and α-tocopherol. Our results are in agreement with those of mentioned authors, who established that genistein was much potent that daidzein in FRAP and
DPPH assays.

The ability of genistein, but not of daidzein, to inhibit the formation of PBN-OOL/-OL spin adducts during catalytic oxidation of soybean oil (Fig. 1b) was in agreement with the cited results of chelating capacity of isoflavones (Mitchell et al., 1998) and with the results of chelating activities of isoflavone aglycones reported by Lee and Cheigh (1997).

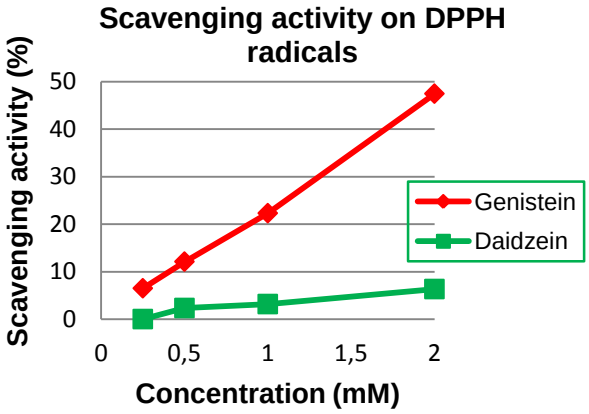

Fig. 2. Scavenging activity of genistein and daidzein on DPPH radicals

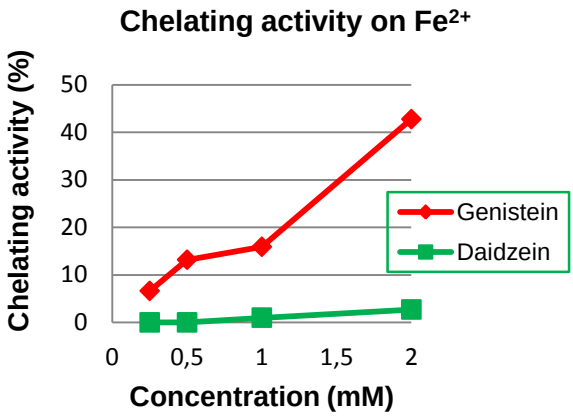

Fig. 3. Chelating activity on Fe^{2+} of genistein and daidzein

The chelating activity of genistein is attributed to the keto group at position 4 and the hydroxyl group at position 5, although these chemical features are not so powerful for chelation as some others (for example, those of quercetin). The structure of daidzein, without the structural predisposition for chelating activity, indicates that it is unable to act as metal-chelator, although it demonstrated extremely weak chelating activity on Fe^{2+} (Fig. 3).

The established antioxidant properties of investigated soybean isoflavones may contribute to the inhibition of lipid peroxidation which occurs during the production, storage and distribution of full-fat soybean foods and feeds.

CONCLUSIONS

Genistein inhibited the thermal oxidation of soybean oil being capable to suppress the formation of PBN-OOL/-OL spin adducts acting as H-donor. In contrast, genistein acted as a chain-breaking antioxidant and a metal-chelator during the catalytic oxidation of soybean oil. It was confirmed that genistein is able to donate H-atom in DDPH test and to chelate Fe^{2+} in chelating activity assay.

Daidzein acted as a very weak chain breaking antioxidant and almost did not exhibit the chelating activity on Fe^{2+}.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31029 “Funkcionalni proizvodi na bazi žita namenjeni osobama sa metaboličkim poremećajima (Functional Cereal-based Products for People with Metabolic Disorder)” financed by the Ministry of Education and Science, Republic of Serbia.

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ANTIOXIDANT PROFILE OF *LAMIACEAE* PLANT EXTRACTS: IMPACT OF ROSMARINIC ACID CONTENT

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ABSTRACT: The aim of this study was to compare the antioxidant properties of five *Lamiaceae* plant extracts using eight different antioxidant assays and to investigate the influence of the rosmarinic acid content on the antioxidant properties of investigated plant extracts. The research includes five plant extracts: *Melissa officinalis*, *Mentha piperita*, *Origanum vulgare*, *Salvia officinalis* and *Thymus serpyllum*. The content of rosmarinic acid in extracts was determined using HPLC-RP-DAD. All extracts were extremely rich in phenolic compounds and contained high concentrations of rosmarinic acid. The highest rosmarinic acid content as well as the best antioxidant properties using most of the methods were obtained for *Melissa officinalis*. The significance and role of rosmarinic acid in the antioxidant properties of *Lamiaceae* plant extracts was confirmed and undoubted, but the contribution of other compounds and their possible cumulative or synergistic action also should not be neglected.
Key words: *antioxidants, Lamiaceae, rosmarinic acid*

INTRODUCTION

There is a great demand in the food industry for compounds that effectively inhibit the oxidation of food components, which is the major cause of food deterioration. These processes may be avoided or delayed by antioxidants. The possible toxicity of the conventional synthetic antioxidants, together with consumers' preference for "natural" products have intensified research of different plant extracts which are known to possess the antioxidant activity (Durling et al., 2007; Krishnaiah at al., 2010). There is an increasing interest for spices and aromatic herbs because of their strong antioxidant properties, which exceed many currently used natural and synthetic antioxidants.
Based on the latest literature, the family *Lamiaceae* is a rich source of phenolic acids. The aim of this study was to investigate the influence of the rosmarinic acid content on the antioxidant properties of *Lamiaceae* plant extracts. Rosmarinic acid is known as one of the most dominant phenolic compound in *Lamiaceae* plants and it is often used as chemotaxonomic marker of this plant family (Zgorka and Glowniak, 2001). The results of this investigation should be helpful in order to better explain the complex pharmacological activity of medicinal plants belonging to the *Lamiaceae* family.

MATERIAL AND METHODS

Reagents, solvents and standards

All reagents and solvents used in the experiments were of adequate analytical grade and were obtained from Fluka (Buch, Switzerland), Kemika (Zagreb, Croatia), Merck (Darmstadt, Germany) and Sigma-Aldrich GmbH (Steinheim, Germany).

Instruments

Spectrophotometric measurements were performed on a UV-Vis double beam Specord 200 spectrometer (Analytik Jena GmbH, Germany), Hewlett Packard UV-Vis spectrophotometer model 8453 (Hewlett Packard, Waldbronn, Germany) and Sunrise basic microplate reader (Tecan GmbH, Austria).

The HPLC system used for separation and quantification of rosmarinic acid was composed by a Varian 330 UV/Vis photodiode array (PDA) detector, a ternary gradient liquid Pro Star 230 pump, a model 500 heater, Star 6.0 chromatography workstation and UltraAqueous C 18 column (250x4.6 mm, 5 mm; Restek; maintained at 30°C). The extracts were filtered through 0.45-mm syringe filters, adequately diluted with MeOH, and directly injected through a 20 ml fixed loop into a C 18 guard column.

Plant material and preparation of plant extracts

Plant material, *Melissa officinalis*, *Mentha piperita*, *Origanum vulgare*, *Salvia officinalis* and *Thymus serpyllum* leaves, produced and distributed by Suban (Samobor, Croatia), were purchased from a local herbal pharmacy.

Dried, pulverized plant material (5 g) was extracted with 250 ml of ethanol/water mixture (80/20, v/v) at 60°C for 60 minutes. After the cooling, the sample was filtered and the residual tissues were washed with solvent (3x10 ml). The extractions were performed in triplicate for each sample, and after the filtration, the three sample extracts were combined in a total extract that was evaporated to the volume of 150 ml, centrifuged and used for further analysis.

Antioxidant methods

The antioxidant properties of plant extracts were evaluated by eight different antioxidant assays. All methods were carried out in triplicate, and the presented data are the means of three measurements.

The Folin-Ciocalteu assay has been used for many years as a measure of total phenolics in natural products, but the basic mechanism is an oxidation-reduction reaction and, as such, it can be considered as another antioxidant method (Prior et al., 2005). Total phenolic concentration in *Lamiaceae* plant ethanolic extracts was determined by the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965; Amerine & Ough, 1980), calibrated against gallic acid standards and expressing the results as mg of gallic acid equivalents (GAE) per l of plant extract.

Total reducing capacity of the samples, FRAP, was estimated following the procedure originally described by Benzie & Strain (1996). A standard curve was prepared using different concentrations of Vitamin C.

Radical-scavenging activity was determined using stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical according to the procedure reported by Katalinić et al. (2010), and using stable 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS) according to the procedure reported by Re et al. (1999). The results were expressed as IC50 values, the concentration of an antioxidant needed to decrease the initial radical concentration by 50%. The IC 50 values were estimated from the graph plotting the inhibition percentage against the extract concentration and were expressed in mg GAE/l of tested sample.

Measurement of superoxide anion scavenging activity of the extracts was based on the method Roback & Gryglewski (1988).

The chelating of ferrous ions by the sample was estimated using the method described by Yen et al. (2000).

The antioxidant activity of sage extracts was determined through the Briggs-Rauscher oscillating reaction (Prenesti et al., 2005; Generalić et al., 2011; 2012). The plant extracts were diluted to a total phenol concentration of 100 mg GAE/l before analysis. The efficiency of the antioxidants was expressed as the inhibition time, in minutes, of the oscillations.

The antioxidant activity of *Lamiaceae* plant extracts in an aqueous emulsion system of linoleic acid and β-carotene was determined according to a method of Moure et al. (2000)

(Terpinc & Abramovič, 2009). The final concentration of plant extract phenolics in the emulsion was 1000 mg GAE/l.

HPLC method

A gradient consisting of solvent A (MeCN), solvent B (H_2O/AcOH 99:1 (v/v)), and solvent C (MeOH) was applied at a flow rate of 1.0 ml/min as follows: from 1% A, 95% B, 4% C at 0 min to 5% A, 85% B, 10% C at 15 min, to 15% A, 35% B, 50% C at 45 min, to 20% A, 5% B, 75% C at 60 min, to 1% A, 95% B, 4% C at 72 min, maintaining 1% A, 95% B, 4% C for 3 min (75 min). The signal was monitored at 280 nm, and rosmarinic acid content was quantified based on the areas of their peaks using external standard calibration curves.

RESULTS AND DISCUSSION

Among the plants reported to have antioxidant activity, *Lamiaceae* plants (mint family) are the most widely used and commercialized. These plants have been used in variety of food preparations since ancient times usually as flavouring agents, but various publications have documented also good antioxidant activity of those extracts or its specific compounds (Petersen & Simmonds, 2003; Generalić et al., 2012).

Good antioxidant properties are result of numerous compounds from diverse chemical groups acting together, including a range of phenolic compounds (Lu & Foo, 2002; Durling et al., 2007; Generalić et al., 2011). The majority of the phenolic acids in mint family plans are exclusively those of caffeic acid derivatives. Although presence of different flavonoids and other phenolics in sage extracts is confirmed, the main antioxidant effect has been reported to relate to the presence of phenolic diterpenes (such as carnosic acid and carnosol), caffeic acid oligomers (known as labiataetannins) and, especially, rosmarinic acid (Fecka and Turek, 2008, Generalić et al., 2011). Flavonoids are widely distributed in *Lamiaceae* plants being mostly present as flavones (apigenin, luteolin and their corresponding 6-hydroxilated derivatives), flavonols (mostly kaempferol and quercetin methyl ethers, narirutin, hesperidin), flavononols (taxifolin) and flavon or flavonol glycosides (Cuvelier et al., 1994; Lu and Foo, 2002, Fecka and Turek, 2008). Among phenolic compounds, the rosmarinic acid (ROA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Figure 1), is usually a dominant phenolic of many species of the *Lamiaceae* family (Petersen et al., 2009). A multitude of biological activities for ROA have already been described and investigated (Petersen & Simmonds, 2003).

The results of this study confirmed the presence of ROA in all investigated *Lamiaceae* extracts in extremely high concentrations. The highest content of ROA was detected in *Melissa officinalis* extract (2413 ± 8 mg/l), what is more than four times higher content than it was found in *Origanum vulgare* extract (Figure 2).

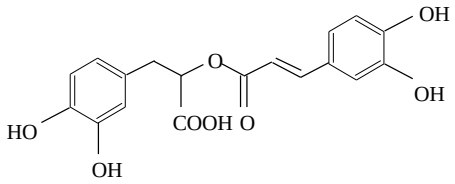

Figure 1. The structure of rosmarinic acid (ROA).

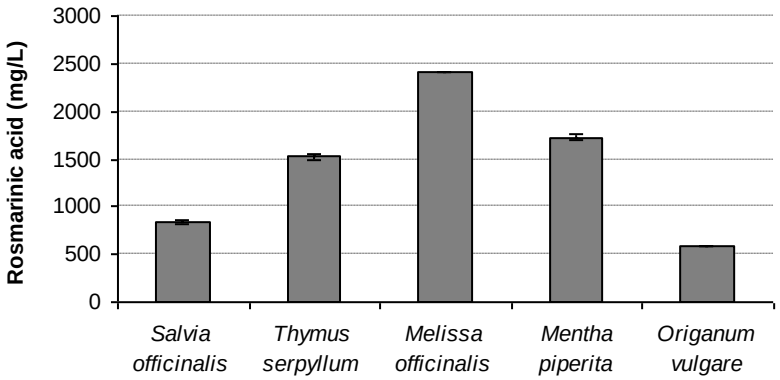

Figure 2. The content of the rosmarinic acid in *Lamiaceae* plant extracts.

The aim of this study was to investigate if there is any connection between the ROA content in plant extracts and its antioxidant properties. The obtained results for all used methods are presented in Figure 3.

As shown in Figure 3a, the total phenolic content in investigated *Lamiaceae* plant extracts was very high and ranged from 4916 to 8998 mg GAE (gallic acid equivalents)/l. The highest content of total phenolic compounds was detected in extracts of *Salvia officinalis* and *Origanum vulgare*. If we compare these results with the results obtained for rosmarinic acid content presented in Figure 2, it can be noticed that the extracts richest with phenolic compounds contain the smallest amounts of ROA. The reason for this could be in the fact that Folin-Ciocalteu reagent is not specific for phenolic compounds only as it can also be reduced by many non-phenolic compounds (Karadag et al., 2009). Compounds like sugars, proteins, aromatic amines, organic acids, inorganic substances, different metal chelators etc. are recognized as interfering substances which can react with FC reagent to give elevated apparent phenolic concentrations (Prior et al., 2005). The reagent therefore measures the reducing capacity of a sample, not just the level of phenolic compounds.

The FRAP values of the investigated plant extracts ranged from 13.2 (*Origanum vulgare*) to 31.8 (*Melissa officinalis*) mM Vitamin C equivalents (VCE) (Figure 3b). It could be noticed that *Melissa officinalis* extract showed the strongest reducing capacity, which was probably related to the presence of high level of ROA in it. Very significant linear correlation was found between the contents of ROA and FRAP values (r = 0.9858). Katalinić et al. (2006) investigated reducing capacity of 70 medicinal plants and also reported best results using FRAP method for *Melissa officinalis* extract, among all tested extracts.

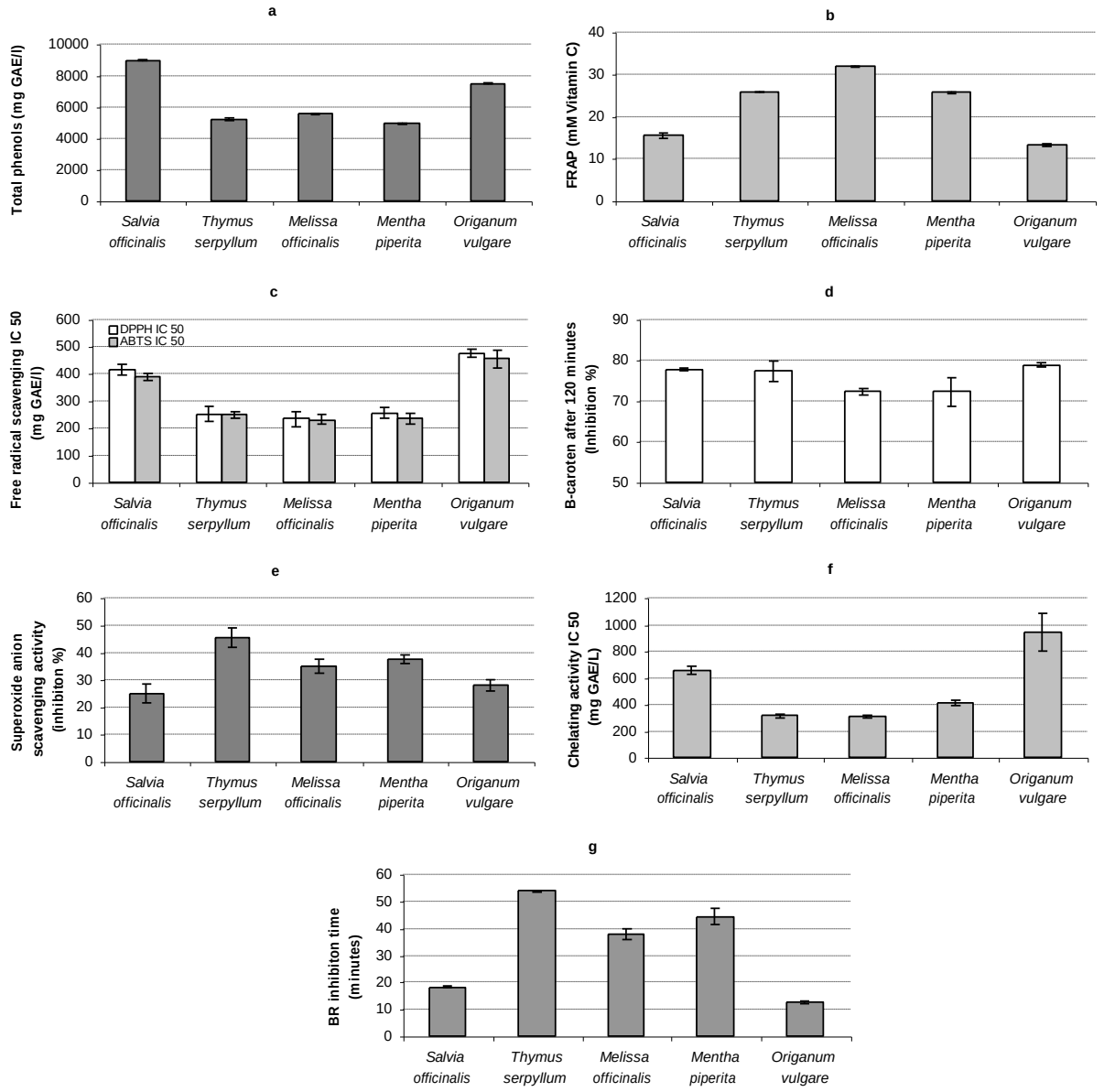

Figure 3. Results for antioxidant properties of *Lamiaceae* plant extracts.
a) Content of total phenols in plant extracts determined using Folin-Ciocalteu assay; b) The ferric-reducing antioxidant power (FRAP) of plant extracts; c) Free radical scavenging activity expressed as values IC 50 (IC 50 is sample concentration needed to decrease the initial free radical concentration by 50%) for DPPH and ABTS (in mg of gallic acid equivalents (GAE)/l of extract); d) Antioxidant activity obtained after 120 min using the B-carotene bleaching assay; e) Superoxide anion scavenging activity of plant extracts expressed as IC 50 value; f) Chelating activity (%) of plant extracts; g) The antioxidant results for the Briggs-Rauscher method, expressed as inhibition time of oscillations

The free radical-scavenging activity of the plant extracts was tested with two synthetic, stable free radicals, $DPPH^{\cdot}$ and $ABTS^{\cdot-}$. The obtained results, expressed as IC 50 values, are presented in Figure 3c. An extremely significant correlation between the IC 50 values of DPPH and ABTS of the extracts was observed (r = 0.9978), and the best results were again obtained for the *Melissa officinalis* extract (DPPH IC 50 = 233 mg GAE/l; ABTS IC 50 = 232 mg GAE/l). Capecka et al. (2005) also investigated free radical scavenging activity of *Lamiaceae* plant extracts. It their study *Melissa officinalis* extract provided the highest antioxidant activity what is in accordance with our results. Negative linear correlation was found between the contents of ROA and IC 50 values obtained by DPPH method (r = -0.9197), as well as by ABTS method (r = -0.9102).

The reactions that occurs in FRAP, DPPH and ABTS methods utilize the same single electron transfer mechanism (ET). This could be reason for good correlation between the ROA content and obtained antioxidant properties (Prior et al., 2005; Badarinath et al., 2010). The position and/or number of OH groups on the benzene ring also affect the extent of direct electron transfer reactions. Rosmarinic acid has four hydroxyl groups and that could be the reason why the extract with the highest share of ROA showed best results.
The best antioxidant activity using superoxide anion assay was obtained for *Thymus serpylum* plant extract (45.49%). This method also provides information on free radical scavenging activity, but it is based on hydrogen atom transfer (HAT) so there is no correlation between obtained results and ROA content.
The antioxidant activity of the *Lamiaceae* plant extracts was also tested as the lipid peroxidation inhibition potential using the B-carotene bleaching method. In the B-carotene/linoleic acid system, the *Origanum vulgare* extract was superior to the other extracts studied. Results of good activity of *Lamiaceae* plants in preventing lipid oxidation were also reported by Su et al. (2007) and Dastalmachi et al. (2008). A negative correlation was found between the contents of ROA and B-carotene inhibition % ($r = -0.8801$).
The results of chelating activity again confirmed that the best antioxidant results were provided for *Melissa officinalis* extracts (KEL IC 50 = 305 mg GAE/l). This is in accordance with results of Dastalmachi et al. (2008). Rosmarinic acid has two *ortho*-dihydroxy groups (catechol structures) (Figure 1), which is the most important structural feature for strong chelating activity of the phenolic compounds (Shan et al., 2005). A negative correlation was found between the contents of ROA and chelating activity ($r = -0.8831$), what confirms this observations.
The results obtained using Briggs-Rauscher oscillating methods are presented in Figure 3g. The inhibition time of the oscillations depends on the free-radical scavenging ability of the added antioxidant compound/mixture (Generalić et al., 2012). The *Thymus serpylum* extract provided the longest inhibition (54 minutes), while the time of inhibition of the *Salvia officinalis* extract as well as *Origanum vulgare* extract was 3-4 times lower.

CONCLUSIONS

The results of this study confirmed that all investigated *Lamiaceae plant* extracts are rich source of rosmarinic acid. The highest content of rosmarinic acid, the best antioxidant properties using FRAP, DPPH and ABTS methods and the best chelating activity were obtained for *Melissa officinalis* plant extract. Single electron transfer mechanism (ET) of these methods and catechol structure of the rosmarinic acid could be reasons for good correlation between the ROA content and antioxidant results. The significance and role of the rosmarinic acid in the antioxidant properties of investigated *Lamiaceae* plant extracts was confirmed and undoubted, but the contribution of other compounds and their possible cumulative or synergistic action should not be neglected.

ACKNOWLEDGEMENTS

We would like to thank the Ministry of Science, Education and Sports of the Republic of Croatia and the Ministry of Education, Science and Sport of the Republic of Slovenia for financing the national (011-2160547-2226) and bilateral BI-CRO-SLO-044-09/10 projects.

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DEVELOPMENT OF A QUINCE SNACK ENRICHED WITH INULIN AND STEVIA

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ABSTRACT: In recent years there is an increased demand for food enriched with physiologically active components. Quinces were selected as a raw material in this study. They belong to the same family of fruits such as pears and apples. The quinces that have been examined had around 20% dry mass, 1.7 % fibers, more than 6 mg vitamin C per 100 g fruit, pectin, antioxidants, and other components. Because of the astringent, tart flavour, quinces are commonly processed into marmalades and jellies, with addition of sucrose to improve their taste. Therefore the purpose of this investigation was to develop a new quince snack product which besides functional properties, will also have improved taste due to the infusion of the alternative sweetener, stevia. This study was carried out to evaluate selected quality attributes of a quince snack enriched with prebiotic (inulin) and alternative sweetener (stevia) accompanied with an antibrowning agent by the use of vacuum impregnation. The porosity of the quince was 0.35 cm^3/cm^3, confirming that its tissue was highly suitable for vacuum infusion. Texture profile analysis was measured on texture analyzer using the compression test that simulates the mastication process. The L*, a* and b* color values were recorded and used to calculate the browning index in all processing steps, in order to obtain the kinetics of the browning reaction. Due to the quince's susceptibility to intensive browning, the kinetics of the browning reaction was necessary to optimize the concentration of the antibrowning agent.
Key words: *quince, inulin, stevia, functional food, snack*

INTRODUCTION

Quince is the fruit of a deciduous tree of the *Rosaceae* family, *Cydonia oblonga* Miller. When ripe, quince fruits impart an agreeable, long-lasting, and powerful flavor. Quince fruit is too acid, astringent, and tough to be consumed fresh. As they are not edible unprocessed because of their very hard, tough, and fibrous consistency, they are often used for preparing jam (Silva et al., 2005). Nevertheless, the consumer's demand for a broader variety of convenient food products has forced the researchers to also investigate the other possibilities of quince processing besides jam.
Vacuum impregnation (VI), has been used as an effective technique in the food industry for enriching porous matrixes and production of new functional foods, due to its advantages: kinetics of transference of fast masses, higher gain of solutes in short times, better color conservation and color improvement in some products and taste and scent conservation of the fresh product (Chiralt et al., 1999). Another potentially great technique with commercial importance is the application of ultrasound (US). Ultrasound waves are supposed to improve porosity of the fruit tissue and enhance the effect of the vacuum infusion. Ultrasonically treated and vacuum impregnated samples can be dried at lower temperatures than the conventional methodology which reduces the probability of oxidation or degradation in the material and therefore their combination can have beneficial effect on the taste, texture and shelf life (Fernandes and Rodrigues, 2007; Fito et al., 2001).
Stevia and inulin, have not been evaluated yet, as potential ingredients, which could be used in quince processing and development of new products. Among the natural sweeteners, stevioside, a sweet glycoside (about 300 times sweeter than sucrose) from *Stevia*

rebaudiana Bertoni, has derived much attention lately (Goyal 2010), and is considered as safe (GRAS) by the FDA. Steviol glycosides can be particularly beneficial to those suffering from obesity, diabetes mellitus, heart disease and dental caries (Ghanta et al., 2007), consumers that could greatly benefit from natural products with low sugar content. Inulin is found widely distributed in nature as plant storage carbohydrates; it is a functional food ingredient that provides unique combination of technological properties. Furthermore, it provides nutritional benefits, which results in better health and attenuation of the risk of many diseases (Nair et al., 2010). Inulin has neutral taste, is colorless, and thereby only minimally influences the organoleptic characteristics of product. The high solubility of inulin enables it to be fortified in many products.
The objectives of this study were to optimize and investigate the effect of stevia and inulin vacuum infusion in fresh quince in order to develop new quince product that can be consumed as dried quince snacks or as a part of dried energy bar or musli formulation.

MATERIAL AND METHODS

Samples and sample preparation

Quince samples were purchased on the market and stored at 1–4°C until use. A few hours prior to use, the samples were left to equilibrate at room temperature. They were peeled manually and cut into 10x10x5 mm cubes after removing the pericarp. Quince cubes were sprayed with 0.25, 0.5 and 1% ascorbic acid solution to inhibit enzymatic browning.

Ultrasound treatment and vacuum impregnation

The quince cubes were dipped separately in three different concentrations of stevia solutions (NOWfoods, USA), 0.75%, 1% and 2% (w/v), and 20% of inulin solution (inulin from dahlia tubers, Fluka, USA). The quince cubes immersed in 250 ml of one of the solutions were exposed to ultrasound (US) treatment for 5, 10 and 15 minutes at three different temperatures (20, 40 and 60 °C). Afterwards, the quince cubes were subjected to vacuum impregnation (VI) step. The vacuum impregnation was carried out for 14 min, where a gradual increase of the vacuum for 4.5 min, was followed by 5 min holding time at vacuum pressure, 100 mbar, and then the gradual release of the vacuum was 4.5 min.

Drying treatment

After the US+VI treatments quince samples were dried at 45°C in a vacuum dryer until constant mass. After the drying, the quince cubes were cooled down to room temperature for 20 min and packed in multi-layered packaging bags. The packaging material was metalized polypropylene and the cubes were packed under the nitrogen atmosphere to avoid moisture absorption, discoloration and spoilage till further analysis. Three replicates were used for each treatment where a replicate consisted of six randomly selected cubes from two quince samples.

Porosity

The bulk volume of fresh quince was measured using the liquid displacement technique. The bulk density, ρb, (g/cm3) was expressed as the ratio of the sample to its bulk volume, while the solid density, ρs, was determined by dividing the mass of the grinded sample by its solid volume. The test was repeated three times. The porosity, Φ, was then calculated as:

$$\Phi = 1 - \frac{\rho_b}{\rho_s}$$

Texture analysis

Texture measurements were conducted by means of a texture analyzer (TA-XT2i of Stable Micro Systems, Godalming, England). To determine the texture of the fresh quince and quince snacks compression test was. This test was conducted using a cylindrical probe (diameter-5 mm; height-25mm). The probe descended at speed of 10 mm/s and compressed the sample at a speed of 1 mm/s up to a distance making 50% deformation. When the compression stroke was completed, the probe abruptly reversed its direction and started the upward move at 10 mm/s. Then a second down and up cycle was run on the same sample. A force-time curve was recorded by the instrument and three textural attributes including hardness, cohesiveness and springiness were calculated.

Measurement of color

To measure the color of fresh quince as well as the color of the treated quince samples, Dr. Lange spectro-color colorimeter was used. The instrument was calibrated against the black and white tile before use. After collecting the three parameters that is L* (lightness), a* (greenness) and b* (yellowness) they were fitted in three equations to calculate the total color change, *ΔE,* and the browning index, *BI*. The experiments were replicated five times.

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}$$

$$BI = \frac{100}{0.17}\left(\frac{a^* + 1.75L^*}{5.645L^* + a^* - 3.012b^*} - 0{,}31\right)$$

RESULTS AND DISCUSSION

Due to the quince's susceptibility to intensive enzymatic browning, the kinetics of the browning reaction of fresh cut quince samples and samples treated with 0.25, 0.5 and 1% ascorbic acid as anti-browning agents, was evaluated to optimize the concentration of the anti-browning agent. The enzymatic browning of the fresh quince followed the 0-order kinetic (Fig 1). As can be seen from the results, the 1% ascorbic acid slowed down the browning process more efficiently than 0.25% and 0.5% ascorbic acid solutions.

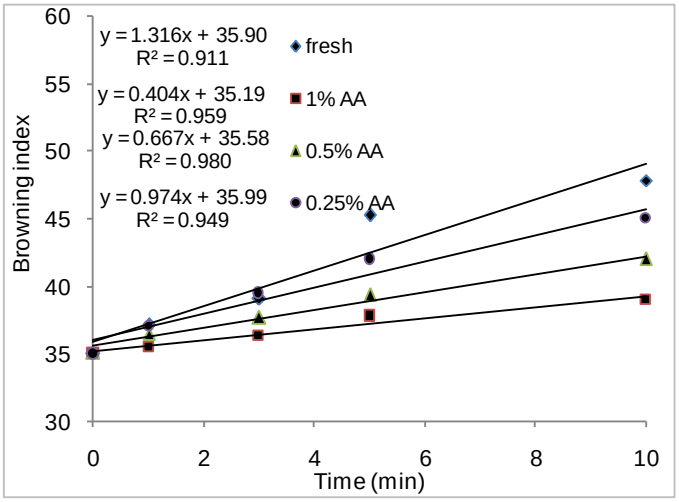

Figure 1. Browning kinetics of fresh and ascorbic acid treated quince

Porosity of the quince samples can be related to the degree of water loss and solute uptake, the fruit's moisture content and the microstructural changes of the tissue during drying. Usually the changes in fruit porosity result in changes of its texture, especially its firmness (Mandala et al. 2005). Therefore, variation in porosity due to the US treatment at temperatures of 20, 40 and 60°C in duration of 5, 10 and 15 minutes was examined (Fig. 2a). Texture profile analysis were also performed (Fig. 2b). The textural changes of the quince

tissue with the change of porosity were compared in order to optimize the US treatment. The US treatment of 10 minutes at temperature of 20°C was chosen as the best condition for pre-treatment of the quince samples. The porosity of those samples was 0.484 cm^3/cm^3, which is 1.4 times higher than fresh untreated quince sample. Even though the porosity of the quince increased with the higher temperature, from the texture profile it was evident that higher temperature led to tissue hardening and loss of springiness and cohesiveness. Furthermore, treatment at this temperature brought development of cooked flavor, which might have negative effect on the sensorial properties of the processed quince cubes.

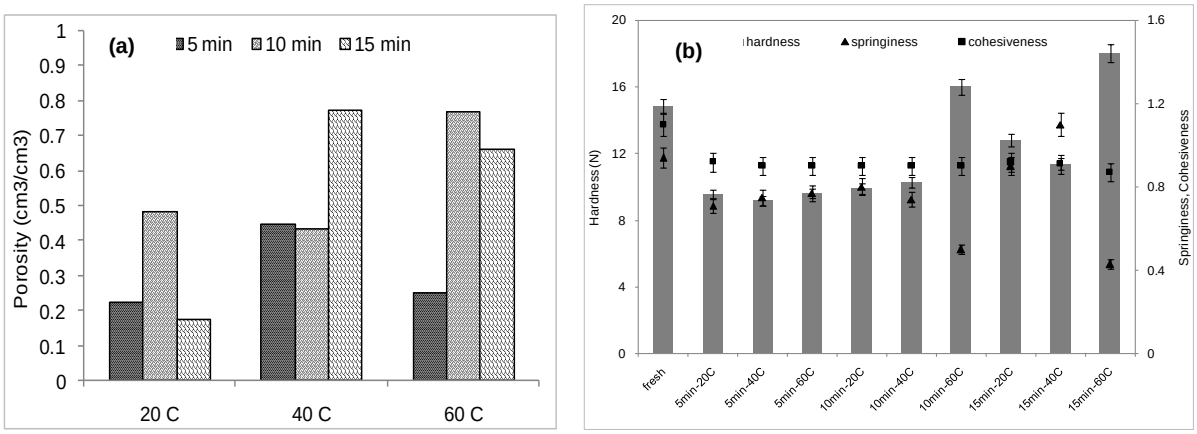

Figure 2. Porosity (a) and textural changes (b) of the quince samples treated with ultrasound

After the optimization of the US treatment, the quince samples were vacuum infused with stevia (1%) and inulin (20%) to investigate the weight gain of the different combination of the pre-treatments (Tab. 1). From the presented results it was noticeable that the combination of the US followed by VI step gave the highest weight gain of the samples. Only VI exhibited slightly lower solute gain, while the application of US after the VI led to loss of the infused solute. Therefore, the further experiments were a comparison between VI and US+VI quince samples.

Table 1. Weight gain of the vacuum infused inulin and stevia in quince tissue

treatment	**weight gain (%)**	
	inulin	**stevia**
Vacuum infusion	9,7	0,85
Ultrasound+Vacuum infusion	10,3	0,98
Vacuum infusion+Ultrasound	6,08	0,56

The textural changes of the quince samples after pre-treatment and after drying are given in Fig.3. The results showed that the pre-treatment did not cause significant change of the tissue firmness. The springiness after the pre-treatment was slightly more pronounced confirming the higher elasticity of the tissue when compared to fresh quince sample. In general, the addition of the US step to the VI treatment increased the springiness of the samples, probably due to cellular restructuring under the ultrasonication. The textural profile of the pre-treated dried samples (Fig. 3b) exhibited tissue hardening after the drying, as expected. The US+VI samples were 2 to 3 times harder when compared to just VI samples. The differences in the firmness between inulin and stevia infused samples were not significant. The springiness of the treated samples after drying followed the same behaviour as after vacuum infusion, meaning that US+VI samples maintained the higher elasticity when compared to just VI samples.

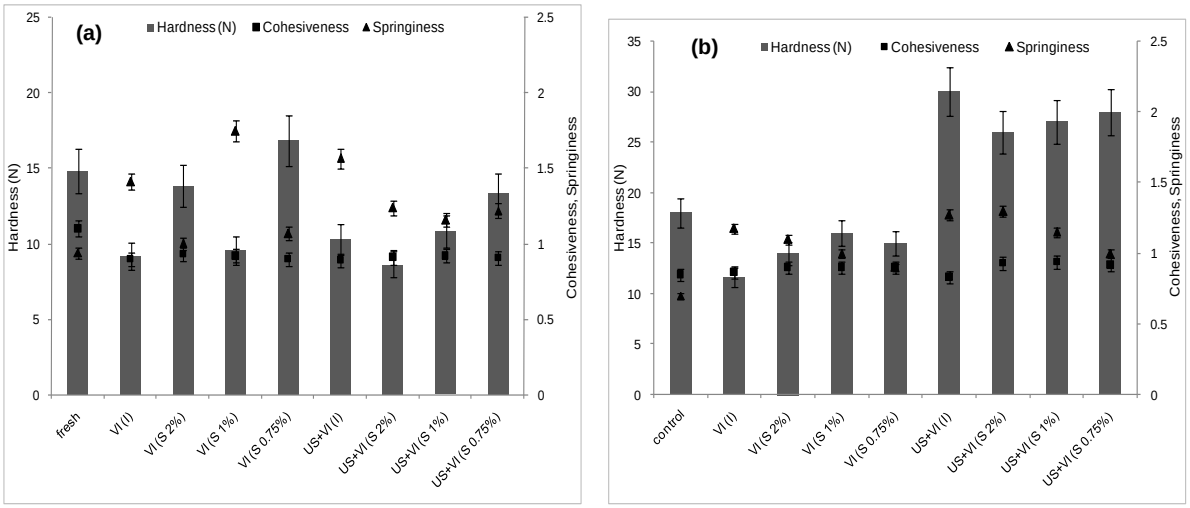

Figure 3. Changes in texture properties of quince samples after pre-treatment (a) and drying (b)

Figure 4 shows the total color change and browning index of the fresh and processed quince samples. As can be seen from Figure 4, the quince enriched with inulin, both VI and US+VI processed, showed similar total color change values as fresh quince, but increased browning index. The stevia infused samples had higher total color change values than the fresh samples and lower degree of browning. These lower values were associated with transparency gained due to air loss, an effect that is produced by the total or partial substitution of the air present in the pores by the impregnation solution (Moreno et al., 2000). The US+VI samples treated with stevia maintained the same browning degree and only inulin treated quince showed significantly higher browning degree when compared to the rest of the samples.

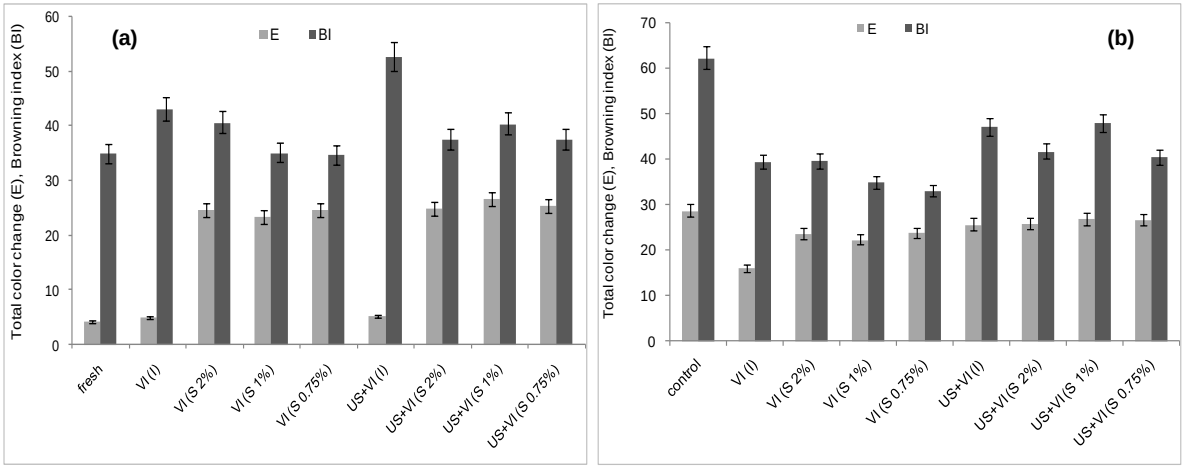

Figure 4. Changes in the surface color of quince samples after pre-treatment (a) and drying (b)

After the drying the pre-treated quince cubes were compared to the color of fresh quince used as positive control and the color of the dried non-treated quince as negative control. It was evident that the pre-treatment led to significantly lower color changes and browning index when compared to the non-treated dried quince. The comparison between the VI and US+VI samples showed that longer processing time (US+VI) ended in higher changes, but still in the acceptable region as proposed by the use of these pre-treating techniques in fruit processing.

CONCLUSIONS

From the above results it was concluded that the combination of ultrasound and vacuum impregnation is a good method for development of functional food and to obtain high quality products. When comparing the results from the experiments, it was evident that the US and VI treatment gave the highest weight gain and maintained the higher springiness. Pre-treatment led to significantly lower color changes and browning index when compared to the non-treated dried quince.

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RELATIONSHIP BETWEEN PHENOLIC CONTENT AND OXIDATIVE STABILITY IN DALMATIAN VIRGIN OLIVE OILS

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ABSTRACT: Olive oil is a prominent source of monounsaturated fatty acids (MUFAs) due to its high oleic content, as well it's a rich source of natural antioxidants (vitamin E, polyphenols such is oleuropein and tyrosol). Its antioxidant content depends on different factors, such is cultivar, fruit ripening stage, climate conditions and olive growing techniques. Oxidative stability of olive oils is related to the ratio of monounsaturated and polyunsaturated fatty acids, and is also influenced by phenolic fraction. The aim of the study presented in this paper was to determine the fatty acid composition, total phenol content and composition of single phenol compounds and their correlation with oxidative stability of examined oils. Fatty acid content was determined by GC technique. Total phenol content was measured colorimetrically, while the single phenol composition was determined using HPLC-DAD/MSD system. Oxidative stability of oils was evaluated by OSI instrument. In this study, in all samples, very good total phenol content was detected, up to 384.33 mg/kg. Among single phenolics, the most representative was hydroxsityrosol and oleuropein aglycon. Oxidative stability data showed very good storage capacity, depending on fatty acid and phenol composition. Oxidative stability of two cultivars was correlated with their oleic/linoleic ratio, while in others it depends also on phenol composition.
Keywords: *olive oil, oxidative stability, phenols, fatty acid composition*

INTRODUCTION

As a central part of Mediterranean diet, virgin olive oil (VOO) is considered to have significant biological and nutritional characteristics. It is obtained directly from the fruit of the olive tree solely by mechanical or other physical means. It is only vegetable oil that can be consumed directly in its raw state, without refinement, and thus contains important nutritional elements (vitamins, antioxidants etc.) It is composed of triacylglycerols (97-98%), minor compounds – partially glycerides, phospholipids and oxidized triacylglycerols- and around 1% of unsaponifiable constituents of varied structure and polarity (Boskou, 1996).
The oxidative stability, sensory quality and health properties of VOO stem from a prominent and well-balanced chemical composition (Bendini et al., 2007). VOO is unique among other vegetable oils due to its high levels of monounsaturated fatty acids (mainly oleic acid) and to the presence of minor components, such as phenolic compounds among others (Cerretani et al., 2004; Deiana et al., 2002; Esti et al., 2004). The content of phenolic compounds is an important factor that influences the quality of VOO (Servilli et al., 2004) particularly because of their potent antioxidant activity and significant contribution to stability of VOOs against oxidation (Tura et al., 2007). The concentration and composition of phenolic compounds VOO are strongly affected by agronomical (Pinelli et al., 2003) and technological factors (Angerosa et al., 1996), such as olive cultivar (Baccouri et al., 2008; Tura et al., 2007), place of cultivation (Cerretani et al., 2006; Blekas et al., 2002), climate, ripening degree (Bonoli et al., 2004), crop season and production process (Oliveras-López et al., 2007). The oxidative stability of VOO depends primarily on the characteristic fatty acid composition (mainly

monounsaturated) (Aparicio and Luna, 2002; Velasco and Dobargenes, 2002) and on the presence of minor components (Baldioli et al., 1996; Oliveras-López et al., 2007) having a marked antioxidant activity, phenolic compounds, improperly referred as polyphenols (Gallina Toschi et al., 2005). These compounds are typical for unrefined oils and thus they posses valuable functional, biological (Visioli et al., 2002) and nutritional function (Del Carlo et al., 2004).

Croatia has a great number (about 40) of autochthonous olive varieties (Bakarić et al., 2007). Among them the main variety is Oblica, which is present in about 70% olive orchards, mostly in Dalmatia region. Variety Oblica has dual character; it's used for oil production and as a table olive. Others important cultivars are: Lastovka, Levantinka, Drobnica, Istarska Bjelica, Buža etc. Certain cultivars have local importance, while others are very difficult to find in productive orchards. The aim of the present study was to evaluate the fatty acid composition and the minor polar compounds of 5 monocultivar VOOs in order to characterized autochtonous VOOs and investigate the oxidative stability in relation to analyzed parameters.

MATERIALS AND METHODS

Five VOO samples, obtained from five olive cultivars, have been chemically characterized. The olive fruit samples were all picked up at the same ripening degree and processed in oil by laboratory plant. Besides basic quality parameters, fatty acid content was determined by GC technique. Determination of fatty acid composition was performed according to the standard method (ISO 5508, 1990). Fatty acid methyl esters (FAME) from the oil samples were obtained by alkaline treatment with 1M KOH in methanol.

The analyses of minor components (phenolic fraction) were also carried out. Liquid–liquid extraction of phenolic compounds from olive oils was performed following the protocol described by Pirisi et al. (1997), modified according to Rotondi et al. (2004). Unless otherwise stated, extractions were performed in three replicates (n=3). Extracts were stored at -18°C before use. Total phenol content was measured colorimetrically according modified method of Gutfinger (1981), while the single phenol composition was determined using HPLC-DAD/MSD system, according to Rotondi et al., (2004). Oxidative stability of oils was evaluated by OSI instrument (Oxidative Stability Instrument), Omnium, Decatur, Il USA. Eight channel OSI instrument was adjusted to 110^{o}C. With air flow of 120 mL/min. Oil stability index (OSI) was measured according to Bendini et al., (2007) in triplicate.

RESULTS AND DISCUSSION

The results for free acidity, peroxide value and UV absorbance show that all analyzed oils were in the category of extra virgin olive oil, according to EC regulations. The fatty acid composition of the analyzed oils obtained from different cultivars showed some differences between them (as showed in table 1), but in all cases met the limits reported in the EC Regulations for olive oils. Fatty acid composition in all samples showed oleic content above 70%. In all samples a stable ratio of oleic and linoleic acid (18:1/18:2) was detected (figure 1), with values close to or higher than 7, with an exception for variety Lastovka that had value 6.38, due to a particularly high content of linoleic acid (11.42%). The monounsaturated fatty acids have great importance because of their nutritional implication and effect on oxidative stability of oils. All analyzed oil samples had content of oleic acid (C18:1) higher than 70%. The highest level of oleic acid was detected in oils from Levantinka variety (over 76%), while the lowest value was found in Lastovka oil. Further, in all oil samples a satisfactory content of palmitic acid (C16:0), the major saturated fatty acid in olive oil, has been detected with media of 12.57%. The content of another important saturated fatty acid, stearic acid (C18:0) had the values within the range 1.26% for Mastrinka oil to 2.19% for oil from Lastovka cultivar.

Table 1. Fatty acid composition (% of total FA) of analyzed olive oils from different cultivars

Fatty acid composition (% of total FA)	**Oil samples**				
	Oblica	Lastovka	Levantinka	Drobnica	Mastrinka
16:0	12. 26 ± 0. 38	12. 32 ± 0. 59	11. 72 ± 0. 13	13. 51 ± 0. 93	13. 02 ± 0. 00
16:1	0. 75 ± 0. 06	0. 75 ± 0. 04	1. 04 ± 0. 24	1. 04 ± 0. 43	0. 76 ± 0. 00
17:0	0. 03 ± 0. 01	0. 10 ± 0. 01	0. 04 ± 0. 00	0. 04 ± 0. 00	-
17:1	0. 08 ± 0. 04	0. 19 ± 0. 06	0. 08 ± 0. 01	0. 07 ± 0. 00	-
18:0	1. 43 ± 0. 51	1. 71 ± 0. 48	1. 90 ± 0. 10	1. 39 ± 0. 74	1. 26 ± 0. 00
18:1	75. 91 ± 1. 41	71. 81 ± 1. 15	76. 17 ± 0. 30	75. 14 ± 0. 34	74. 39 ± 0. 00
18:2	9. 66 ± 0. 85	11. 63 ± 0. 29	6. 78 ± 0. 19	7. 93 ± 0. 04	10. 21 ± 0. 00
18:3	0. 66 ± 0. 07	0. 66 ± 0. 04	0. 66 ± 0. 03	0. 55 ± 0. 35	0. 34 ± 0. 00
20:0	0. 39 ± 0. 06	0. 48 ± 0. 09	0. 53 ± 0. 11	0. 40 ± 0. 00	0. 02 ± 0. 00
20:1	0. 36 ± 0. 03	0. 38 ± 0. 11	0. 40 ± 0. 08	0. 41 ± 0. 00	0. 02 ± 0. 00
18:1/18:2	7. 92	6. 18	11. 24	9. 47	7. 28

Results are given as mean ± standard deviation (n = 3)

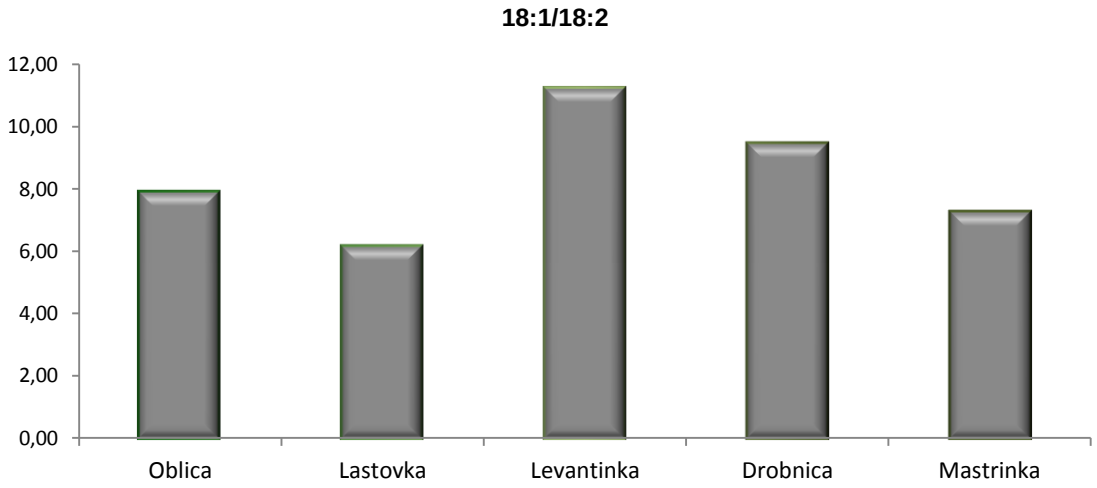

Figure 1. 18:1/18:2 ratios in examined oil samples

The amount and composition of the polyphenols in olive oil was already shown to depend on olive cultivar (besides the agricultural practice and the degree of ripening, too) (Montedoro et al., (1992); Carrasco-Pancorbo et al., 2005). It is shown that in monovarietal oils from Croatian cultivars total polyphenols content have mostly satisfied values, reaching much high content in samples grown in north Adriatic regions (Koprivnjak et al., 1998).
All analyzed VOOs in this study had a total phenol content between 134.4 and 394.40 mg/kg, as well as different composition of individual phenolic compounds, depending on the olive cultivar (Table 2). Among single phenolic compounds, the most representative was hydroxsityrosol and oleuropein aglycone.

Table 2. Total phenols and phenolic composition and proportion of compounds (mg/kg)

		Oil samples				
		Oblica	Lastovka	Levantinka	Drobnica	Mastrinka
Total phenolic content* (mg/kg)		315.57 ± 28.58	286.55 ± 42.86	195.36 ± 56.88	354.72 ± 39.98	254.13 ± 34.68
Phenol compounds (mg/kg)**	**HYTY**	39.30 ± 1.37	132.28 ± 9.05	43.25 ± 2.82	117.73 ± 2.86	55.28 ± 0.18
	TY	10.73 ± 1.09	26.54 ± 4.50	29.76 ± 4.46	98.52 ± 5.25	33.48 ± 0.29
	DOA	45.04 ± 0.02	216.54 ± 10.11	136.43 ± 16.94	144.72 ± 4.02	133.87 ± 2.76
	PIN	20.43 ± 0.93	43.58 ± 6.00	28.73 ± 6.98	39.76 ± 12.57	34.28 ± 0.49
	DLA +AcPin	121.40 ± 4.48	187.65 ± 12.80	186.75 ± 2.84	212.45 ± 3.64	132.93 ± 3.25
	OA	22.52 ± 0.89	257.43 ± 15.30	136.43 ± 11.95	87.29 ± 5.80	122.86 ± 2.79
	LA	8.72 ± 1.26	65.36 ± 2.62	24.63 ± 5.74	23.56 ± 5.73	46.29 ± 3.26
	LU	52.40 ± 4.34	76.85 ± 8.34	42.15 ± 1.54	39.58 ± 7.50	45.69 ± 1.01
	AP	13.84 ± 2.47	26.43 ± 7.56	18.47 ± 7.32	29.86 ± 6.00	15.35 ± 0.22

** measured colorimetrically according modified method of Gutfinger (1981)*
***(1) HYTY – hidroxytyrosol; (2) TY – tyrosol; (3) DOA – decarboxy methil oleuropein aglycone; (4) PIN – pinoresionl; (5) DLA +AcPin – decarboxy methil ligstroside aglycone + acetoxypinoresinol; (6) OA – oleuropein aglycone; (7) LA – ligstroside aglycone; (8) LU – luteoline; (9) AP - apygenine*

Oxidative stability data (figure 2) showed very good storage capacity, depending on fatty acid and phenol composition. Low OSI level for Oblica oil seems to be correlated with 18:1/18:2 ratio, and OSI value for Lastovka to its total phenol content. The highest OSI value was detected for oils from Drobnica and Mastrinka.

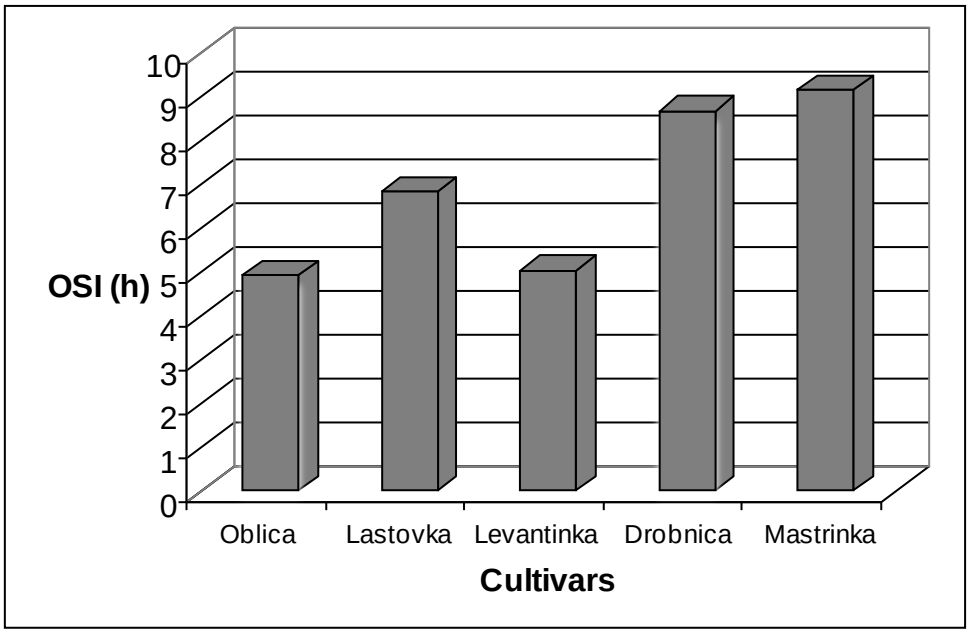

Figure 2. Oil stability index (OSI) for some analyzed olive oils

CONCLUSIONS

In all samples in this study very good total phenol content was detected. Oil from Drobnica variety had the highest level of total phenols, same as the pretty high level of 18:1/18:2 ratio. Thus it can be concluded that this oil has good oxidative stability according to those parameters. Oxidative stability of two cultivars Lastovka and Drobnica was correlated with their oleic/linoleic ratio, while in others it depends also on phenol composition. The best oil stability was attested in VOOs with higher level of total phenols and also higher 18:1/18:2 ratios. Based on the obtained results, further investigations will be focused on determination the correlation between single phenols and oxidative stability of autochthonous VOOs.

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INFLUENCE OF FERTILIZERS AND GROWTH REGULATORS ON NUTRITIONAL QUALITY OF *ALLIUM CEPA* IN DIFFERENT STAGES OF MORPHOGENESIS

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ABSTRACT The aim of this paper was to study the effect of growth regulators in connection with diversified fertilization (concerning the form, the doses and kind of fertilizer) on the nutrients content (Vitamin C and protein) in Allium cepa (*onion)* culture, in different stages of morphogenesis. Field trials were conducted during the early seasons of 2011, on a black Chernozem soil. The experiment was a randomized complete block design with four replications. The experimental site was prepared in 28 beds of 1 x 1 m^2 each in size and the variants used were: $N_0P_0K_0$, $N_{60}P_{45}K_{45,}$ $N_{90}P_{60}K_{60,}$ $N_{120}P_{60}K_{60}$, $N_{60}P_{60}K_{60}$ + Aqzyme 1 l/ha, $N_{60}P_{60}K_{60}$ + 2 x Pervaide 1 l/ha, Manure 20 t/ha.
The protein content analyses were performed in the Laboratory of Soil Science and Plant Nutrition, Faculty of Agriculture, USAMVB Timisoara.
The protein content % was determined with the help of Kjeldahl unit (Velp Scientific 127) and Vitamin C (mg/100 g) content was done according to STAS 6182/15-87.
NPK fertilizers dose not influence the Vitamin C accumulation with highest value being registered in variant v2 – $N_{60}P_{45}K_{45}$, in all morphogenesis stages, 13 mg/100 g (1st stage), 14 mg/100g (2nd stage) and 18 mg/ 100g (3rd stage). Growth regulators and manure does not influence the vitamin C accumulation**.**

INTRODUCTION

Vegetables are a great source of essential vitamins and minerals in the everyday diet. Quality can be characterized by functional values that can be measured or analyzed. The chemical composition and content of nutrients are important for the human diet and determine the nutritional quality of a product (Hauffman, S. and Bruce, A, 2002).
Best quality onion can be produced through application of well balanced fertilizers. Research work has been done on the base of NPK in different soil types and in various climatic conditions, but very limited work has been reported on various sources of fertilizes for a certain nutrient (Ghulam N. et al., 2010).
Onions start bulb formation when the day length is of the proper duration. Different varieties of onions require different day lengths to initiate bulbing. Onions have a high demand for micronutrients such as copper, manganese, molybdenum and zinc.
Fertilization is the most important and controllable factor affecting nutritional value of vegetables. The type and value of fertilizer and the level of application directly influence the level of plant available nutrients and indirectly influence plant physiology and chemical content (Griepentrog H. and Porter J., 2002).
A lot of studies were carried about the differences in vegetables nutrient levels, grown at various levels of nitrogen fertilizer. These studies compare various levels of artificial, organic and mixed fertilizer. However, very few studies compare the effect of fertilizer on nutritional quality of vegetables in organic and conventional production.
Of the major nutrients, nitrogen (N) is often required in the greatest quantity by crops, primarily for vigor and yield. Nitrogen plays a key role in chlorophyll production and protein synthesis (Moigradean Diana et al., 2007).
Proteins are nitrogenous organic substances, composed of substances with plastic role for forming tissue and regeneration. Proteins as macromolecular substances are substances made of amino acids. Proteins have a very wide variety of compounds and they are

practically very difficult to classify and determine as entity substances. It therefore appeals to the determination of total protein (the amount of protein). These substances contain carbon, hydrogen, oxygen and nitrogen always (at a rate of 16%) (Dima D et al., 1991).
Vitamin C is required for the synthesis of collagen, an important structural component of tendons, bones, teeth, blood vessels and muscles. The vitamin also synthesizes a neurotransmitter called norepinephrine. It also plays a role in the fat transport system of cells and cholesterol metabolism (prevention of gallstones). Vitamin C is a powerful antioxidant that assists the body in contesting viral infection, bacterial infections and toxicity (http://www.lenntech.com/vitamins/vitamin-c.htm). A deficiency leads to scurvy, which was the leading cause of death among sailors on ocean vessels from ancient times up until the 18th century. Scurvy is still widespread today in developing countries. In France there does not appear to be major problems in Vitamin C intake among children and adolescents. However, nearly 20% of adults (and especially men) have an intake level of less than two thirds the recommended allowance of Vitamin C. There is no real risk of excess Vitamin C. Only exceptionally high doses that are not possible with a regular diet are potentially toxic (http://www.fondation-louisbonduelle.org/france/en/know-your-vegetables/health-and-nutrients/vitamine-c.html#axzz1sHlTNpRV).
The aim of this research was to determine the effect of growth regulators in connection with diversified fertilization (concerning the form, the doses and kind of fertilizer) on the content of nutrients (Vitamin C and protein) compositions of onion culture, in different stages of morphogenesis.

MATERIALS AND METHODS

Field trials were conducted during the early seasons of 2011, on a black Chernozem soil. The experiment was a randomized complete block design with four replications.
The experimental site was prepared in 28 beds of 1 x 1 m^2 each in size and the variants used were: $N_0P_0K_0$, $N_{60}P_{45}K_{45}$, $N_{90}P_{60}K_{60}$, $N_{120}P_{60}K_{60}$, $N_{60}P_{60}K_{60}$ + Aqzyme 1 l/ha, $N_{60}P_{60}K_{60}$ + 2 x Pervaide 1 l/ha, Manure 20 t/ha. The fertilizers used were NPK complex type 16:16:16, and NH_4NO_3. De Stuttgart variety was used as onion test crop. Weeds were controlled by hoeing and herbicide Dual Gold application.
Aqzyme growth regulator contains Fungi enzymes (99%), copper (0.05%), iron (0.10%), manganese (0.05%), zinc (0.05 %). The state of macro and micronutrients of soil supply was proper, for phosphorus (P) registered 121 mg/kg, the range for vegetables grown in the field, is between 108.1 – 144.0 according to studies of literature [8]. Nt % soil supply state was medium 0.185 %, framing in range 0.141 – 0.270 %, quoted in other studies (Budoi, Gh., 2004).
The onion samples were collected in 3 major stages of culture development: green onion stage *(1st stage of morphogenesis),* beginning of bulb formation *(2nd stage of morphogenesis) and* full maturity *of plant* (*3rd stage of morphogenesis).*
The protein content analyses were performed in the Laboratory of Soil Science and Plant Nutrition, Faculty of Agriculture, USAMVB Timisoara.
The Nt % was determined with the help of Kjeldahl mineralization - distillation unit (Velp Scientific 127) digested in H2SO4 distilled and titrated with 0.1M NaOH. The amount of protein is calculated by the following relationship: *Total Protein content (%) = 6.25 x Nt (%),* where: 6.25 namely 100/16 is the coefficient of conversion into protein. For vitamin C (mg/100 g) determination in onion samples was used 2,6-diclorfenolindofenol, method, according with STAS 6182/15-87. Ascorbic acid content was estimated titrimetrically by 2,6-Dichlorphenolindophenol sodium. 5 mL of vegetable extracts was diluted with 10 mL water, ad 1 mL HCl 1N and was titrated with 1 mM solution 2,6- Dichlorphenolindophenol sodium to pink color (Gergen I., 2004).

RESULTS AND DISCUSSIONS

The influence of mineral and organic fertilizers and growth regulators on crude protein content and vitamin C accumulation is presented in tables 1-2.

Table 1. Vitamin C (mg/100g) content in onion samples in all stages of plant development

Experimental variants	Vitamin C [mg vit.C/100g]		
	1st stage of morphogenesis	*2nd stage of morphogenesis*	*3rd stage of morphogenesis*
v_1 - $N_0P_0K_0$	8±*0.45*	9 ±*2.65*	11±*1.87*
v_2 – $N_{60}P_{45}K_{45}$	**13±*1.37***	**14±*0.45***	**18±*2.13***
v_3 – $N_{90}P_{60}K_{60}$	12 ±*2.41*	13 ±*3.19*	15 ±*0.92*
v_4 – $N_{120}P_{60}K_{60}$	11 ±*1.65*	12 ±*2.67*	14 ±*1.44*
v_5-$N_{60}P_{60}K_{60}$ + Aqzyme 1 l/ha	9 ±*0.45*	13 ±*1.22*	15 ±*2.01*
v_6-$N_{60}P_{60}K_{60}$ + 2 x Pervaide 1 l/ha	10 ±*2.40*	11 ±*1.33*	14 ±*1.15*
v_7 – Manure 20 t/ha	11 ±*2.88*	13±*3.14*	16 ±*0.72*
Cv%	0.162	0.138	0.145

Results for Vitamin C (%) (1st; 2nd; 3rd morphogenesis stages) are given as mean of 4 determinations.

Experimental variants ordered from lowest to highest fertilizers doses showed wide differences between culture morphogenesis stages. The Vitamin C content of onions increases as the culture forward in vegetation towards full maturity. Vitamin C content, for onion, ranged from 8.0-13.0 mg/100g fresh matter, in green onion, in the young plants, highest content was found in $N_{60}P_{45}K_{45}$ fertilization rates and lowest in control samples.
NPK fertilizers dose not influence the Vitamin C accumulation highest value being registered in variant v_2 – $N_{60}P_{45}K_{45}$, in all morphogenesis stages, 13 mg/100 g (1st stage), 14 mg/100g (2nd stage) and 18 mg/ 100g (3rd stage) (table 2). Growth regulators and manure does not influents the vitamin C accumulation. The results are in accordance with other studies that show a content of Vitamin C in onions of 10 mg /100g. (http://www.lenntech.com/vitamins/vitamin-c.htm)
In Romania were done similar studies in tomatoes culture *Lycopersicum esculentum.* Moigradean D. et al., in 2007, studied Vitamin C content in tomatoes samples fertilized with different NPK doses. The results showed that the highest content in Vitamin C was determined in variant with moderated fertilizer dose ($N_{45}P_{45}K_{45}$) and not in variant with highest NPK dose ($N_{120}P_{60}K_{60}$).

Table 2. Protein content (%) in onion culture samples in different morphogenesis stages

Experimental variants	Protein (%)		
	1st stage of morphogenesis	*2nd stage of morphogenesis*	*3rd stage of morphogenesis*
v_1 - $N_0P_0K_0$	2.44±*0.76*	2.84±*1.05*	2.87±*1.13*
v_2 – $N_{60}P_{45}K_{45}$	2.56±*0.67*	3.06±*0.98*	3.24±*1.54*
v_3 – $N_{90}P_{60}K_{60}$	3.22±*1.21*	3.25±*1.53*	3.83±*2.16*
v_4 – $N_{120}P_{60}K_{60}$	**4.75±*1.75***	**4.88±*2.33***	**5.07±*0.99***
v_5-$N_{60}P_{60}K_{60}$ + Aqzyme 1 l/ha	3.06±*2.05*	3.30±*2.12*	3.44±*1.41*
v_6-$N_{60}P_{60}K_{60}$ + 2 x Pervaide 1 l/ha	3.14 ±*1.80*	3.24±*1.75*	3.35±*2.35*
v_7 – Manure 20 t/ha	3.58 ±*1.65*	3.65±*0.78*	3.86±*2.09*
Cv%	0.236	0.194	0.192

Results for crude protein content % (1st; 2nd; 3rd morphogenesis stages) are given as mean of 4 determinations.

Protein content (%) in the early stages of vegetation, for *Allium cepa* culture, in all experimental variants, showed a lower content ranging between 2.44 - 4.75%, values that increase with plant maturity (2.84 - 4.88 % - beginning of bulb formation) or 2.87 - 5.07% at physiological maturity of onions (Table 1).
This is explained by the fact that once plant completes phases of plant growth the maturity, nitrogen needs increase and thus protein content, indispensable for the development of onion culture.
For 2nd morphogenesis stages (beginning of bulb formation of onion culture) the variation limits of protein content are frame between 2.84 – 4.88% (Table 2).
The quality attributes increased as the N rates increased with the highest contents recorded at 120 kg N/ha of fertilizer application. Total crude protein content (%) in green onion, in the 1st morphogenesis stage, ranged from 2.44% to unfertilized control variant to 4.75% to variant 4 fertilized with 120 kg N (Table 2).
Nitrogen is an essential element for healthy plant growth, being a constituent of proteins and chlorophyll. In all morphogenesis stages, the highest protein content was determined at the application of fertilizers in a dose of $N_{120}P_{90}K_{90}$. There is a proportional increase of the protein content of onions with the increasing dose of nitrogen fertilizer applied. Similar results were found by Westerveld et al., in 2006. Onions absorb nitrogen throughout the vegetation period. The amount of nitrogen absorbed is reduced to seeds germination, but grows in phenophases were the main vegetative organs are formed. Presence of phosphorus and potassium applied as fertilizer favors nitrogen accumulation. High nitrogen content stimulates leaf growth at the expense of root development and yield, and also delays harvesting. Very lush leaf growth may also promote the development of diseases, such as Sclerotinia white mould (Alexa E., 2088).
The limits of variation of crude protein content (%) at full maturity of plants, frame between 2.87% (v1) – 5.07 % (v4) (Table 2).

CONCLUSIONS

- During the early morphogenesis stages of vegetable species studied, the values of Vitamin C (mg/100g) and protein (%) were lower, but during culture development the content increases.
- NPK fertilizers dose not influence the Vitamin C accumulation, with highest value being registered in variant v_2 – $N_{60}P_{45}K_{45}$, in all morphogenesis stages, 13 mg/100 g (1st stage), 14 mg/100g (2nd stage) and 18 mg/ 100g (3rd stage).
- Growth regulators and manure does not influence the vitamin C accumulation.
- Protein content (%) in the early stages of vegetation, for *Allium cepa* culture, in all experimental variants, showed a lower content ranging between 2.44 - 4.75%, values increase with plant maturity (2.84 - 4.88 % - beginning of bulb formation) or 2.87 - 5.07% in physiological mature onions.

ACKNOWLEDGEMENTS

This work was published during the project “Postdoctoral School of Agriculture and Veterinary Medicine", POSDRU/89/1.5/S/62371,co-financed by the European Social Fund through the Sectorial Operational Program for the Human Resources Development 2007-2013.

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UTILIZATION OF WITHDRAWN BREAD AND CORN MEAL MIXTURES FOR NEW FEED PRODUCTION

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ABSTRACT: There is an evident problem with withdrawn bread in Serbia, in terms of quantity, environmental and health aspects. There is no organized way for withdrawn bread collecting and distributing for further processing. The absence of these actions contributes to the increasingly polluted environment, and creates problems in the food chain. This paper contributes to solving problem with withdrawn bread using extrusion of the mixtures of corn meal and withdrawn bread (50:50 and 60:40) moisturized up to 18% prior to extrusion. Extrusion of these mixtures was carried out in a single screw extruder (Metal-matic Beočin, Serbia) at temperature of 95-105°C. The results confirm that the obtained products are safe concerning microbiology and mycotoxicology. The total count of molds and microorganisms is below the maximum allowable concentration indicating that the produced extrudates could be used as safe feeds.
Key words: *withdrawn bread, corn meal, extrusion, health and safe food*

INTRODUCTION

In a survey conducted in 45 municipalities in Vojvodina, an analysis of the current situation regarding the type and quantity of withdrawn bread was carried out with the aim to define the sources of its formation and suggets solutions to overcome the existing problem. Withdrawn bread that satisfies, hygienic and microbiological criteria for use in food animals may be raw material quality .

Based on surveys of the Institute of Food Technology in Novi Sad (FINS) made in the period January - March of 2010., the quantity of withdrawn bread varies between 5-10% (Nježić et al., 2010).

It was concluded that approximately 50,000 loaves (500 g) from one million loaves produced in Vojvodina per day are not used. Depending on the health safety withdrawn bread can be considered either a very attractive raw material or a major problem (Nježić et al., 2010).

The solution to increased food production for humans and animals is the application and use of new technologies in biotechnology and bio-industry. New technological procedures aimed at increasing the nutritional value of food intended for human consumption and animals, as well as evaluation of secondary food products and primary agricultural production. Today, thermal processing of oilseeds and cereal grains like toasting, extrusion, micronization, microwave treatment and dielectric heat treatment (Marsman et al., 1998; Sakač et al., 2001) are in use, but in Serbia extrusion process is the most experienced (Filipović et al.; 2008, Sakač et al., 2006).

Thermal treatments have to be optimized to decrease the content of thermolabile antinutrients to acceptable level and to increase the digestibility of some nutrients (oils and carbohydrates) as well as to improve the sensory properties and microbiological characteristics of the final product. (Filipović et al., 2007; Zhou and Erdman, 1995).

Corn extrusion in the feed production contributes to a better valorization of food in animal nutrition (Filipović et al., 2008).

Along with the reduction of the antinutrients, it is necessary to preserve thermolabile nutritionally valuable components and the process requires a compromise between these two efforts (Jensen et al., 1995).

Extrusion leads to changes in the carbohydrate complex of corn meal and bread crumbs, or a decrease in starch content due to its degradation to dextrins. Such changes contribute to *in vitro* and *in vivo* digestibility of starch, since starch gelatinization provides increased availability of enzymes that break down or to inactivation of amylase inhibitors (Douglas et al., 1990).
The aim of this study was to determine the effect of extrusion on the quality of the mixture of corn meal and bread crumbs.

MATERIALS AND METHODS

Starch content, as well as total and reducing sugars were determined by the Regulation on the physical and chemical methods of analysis for quality control of grain, milling and bakery products, pasta and deep frozen dough (Službeni list SFRJ, 1988). Bulk density was determined by the Regulations on methods of sampling and methods of performing physical, chemical and microbiological analysis of animal feed (Službeni list SFRJ, 1987). The basic chemical composition (moisture, crude protein, crude fat and ash) of corn meal and bread crumbs mixture is determined by AOAC methods
Prior to extrusion moisture of corn meal (moisture 12%) and bread crumbs (50:50 and 60:40) was adjusted to of 18% in a premixer. Extrusion was carried out in a extruder which capacity is 900 kg/h. The installed electric power of extruder and the screw dispenser was 100 kW and 1.1 kW, respectively. Extrusion temperature was 90 and 95 °C, and die diameter was 7.5 mm.
Total count, the number of yeasts and molds and isolation and identification of *Salmonella* and Sulphite-reducing *Clostridia* were determined by the Regulation on the method of the microbiological analysis of food and super analysis of animal feed (Službeni list SFRJ, 1980).
For determination of the presence of coagulase positive *Staphylococci*, Proteus species and *Escherichia coli* modified method of sample preparation. 50 g of sample was weight in the test flask and poured Erlenmajer to 450 ml of sterile nutrient broth prepared. Prepared sample is gently homogenized and incubated for 24 h at 37 °C. Isolation and identification is carried out, according to the method of the microbiological analysis and superanaysis of food (Službeni list SFRJ, 1980).

RESULTS AND DISCUSSION

Chemical composition and particle size distribution of corn meal, are presented in Table 1 indicating that the feed is characterized with 75.23% of starch, protein 6.88% and 1.14% fat, and with energetic value of 1544 kJ/100 g. The particle size distribution shows that only 9.8% of particles were greater than 550 microns and 81% of particles possesed the diameter greater than 250 microns. Bulk density of investigated corn meal was 654 kg/m^3. Table 2 shows the content of microorganisms and mycotoxins in corn meal.

Table 1. Chemical composition, particle size distribution and bulk density of corn meal

Quality parameters	Content (%)
Moisture	13.36
Ash	0.24
Crude protein	6.88
Total sugar	2.23
Reducing sugars	0.49
Starch	75.23
Fat	1.14
Energetic value	**kJ/100 g**
Calorimetric determinated eneregetic value	1544
Particle size distribution	**Content (%)**
Sieve fractions over 550 µm	9.8
Sieve fractions over 250 µm	81
Sieve fractions under 63 µm	9.2
Bulk density	654 kg/m^3

Table 2. Microorganisms and mycotoxins content in corn meal

Microorganism	**dilution**	**number**
Salmonella spp	in 50 g	not detected
Coagulase positive *Staphylococci*	in 50 g	not detected
Sulphate-reducing *Clostridia*	in 1 g	not detected
Proteus varieties	in 50 g	not detected
Escherichia coli	in 50 g	not detected
Total number of yeasts	in 1 g	not detected
Total number of fungi	in 1 g	80
Total number of microorganisms	in 1 g	500
Mycotoxin content	**µg/kg**	
Aflatoxins B1+G1+B2+G2	<3	
Ochratoxin A	<10	
Zearalenon	<25	

From the data shown in Table 2 it is evident that *Salmonella spp.*, Coagulase positive *Staphylococci*, sulphate-reducing *Clostridia*, *Proteus* species, *Escherichia coli* and yeasts were not found in investigated corn meal. Total number of fungi and microorganisms in 1 g was 80 and 500, respectively. Mycotoxicology shows that the aflatoxin, ochratoxin A and zearelenon content was less than 3 µg/kg, 10 µg/kg, and 25 µg/kg, respectively indicating that this is according to the Regulation (Pravilnik o metodama vršenja mikrobioloških analiza i superanaliza životnih namirnica, 1980). Table 3 shows the chemical composition and particle size of the bread crumbs.

Table 3. Chemical composition, particle size distribution and bulk density of bread crumbs

Quality parameters	**Content (%)**
Moisture	12.87
Ash	2.24
Crude protein	11.44
Total sugar	2.72
Reducing sugars	2.08
Starch	63.34
Fat	3.18
Energetic value	**kJ/100 g**
Calorimetric determinated eneregetic value	1589
Particle size distribution	**Sadržaj (%)**
Sieve fractions over 550 µm	45.6
Sieve fractions over 250 µm	40.2
Sieve fractions under 63 µm	14.2
Bulk density	415 kg/m^3

Bread crumbs are a food product obtained by subsequent treatment of withdrawn bread.This product contains 63.34% starch, 11.44% protein and 3.18% fat, with the energy value of 1589 kJ/100 g, thus it is high quality energy feed. The content of microorganisms and mycotoxins in bread crumbs is shown in table 4.

Table 4. Microorganisms and mycotoxins content in bread crumbs

Microorganism	**dilution**	**number**
Salmonella spp	in 50 g	not detected
Coagulase positive *Staphylococci*	in 50 g	not detected
Sulphate-reducing *Clostridia*	in 1 g	not detected
Proteus varieties	in 50 g	not detected
Escherichia coli	in 50 g	not detected
Total number of yeasts	in 1 g	not detected
Total number of fungi	in 1 g	30
Total number of microorganisms	in 1 g	30
Mycotoxin content	µg/kg	
Aflatoxins B1+G1+B2+G2	<3	
Ochratoxin A	<10	
Zearalenon	<25	

Microbiological and toxicological tests show that bread crumbs are hygienically safe, Table 4. The total number of fungi and microorganisms are 30 per 1g, while aflatoxins, ochratoxin A and zearalenone were less than 3 µg/kg, 10 µg/kg, and 25 µg/kg, respectively. Table 5 shows the chemical composition and particle size distribution of the extruded mixture of bread crumbs and corn meal.

Table 5. Chemical composition, particle size distribution and bulk density of extruded mixtures of corn meal and bread crumbs

Quality parameters	**Content (%)**	
	Extruded mixture of corn meal:bread crumbs, 50:50	Extruded mixture of corn meal:bread crumbs, 60:40
Moisture	8.40	8.53
Ash	9.25	9.81
Crude	1.25	1.03
Total	5.20	5.93
Reducing sugars	3.46	3.40
Starch	69.8	65.36
Fat	2.64	2.12
Energetic value	**kJ/100 g**	
Calorimetric determinated eneregetic value	1635	1642
Particle size distribution	**Content (%)**	
Bulk density	119 kg/m^3	92 kg/m^3

From the obtained results a reduced moisture content compared to the starting raw materials reduction in starch, and increased total and reducing sugars is evident, as a result of thermal decomposition of starch, resulting in increased digestibility and improved utilization of starch (Douglas et al., 1990; Zhou and Erdman, 1995). Increased sweetness, i.e. change the sensory properties is a consequence of chemical changes in starch. Change is evident in the decreased bulk density of extrudates tha are in the range 92 - 119 kg/m3.
The content of microorganisms in extruded corn meal and bread crumbs mixtures is shown in Table 6.

Table 6. Microorganisms in mixtures extruded corn meal and bread crumbs

Microorganism	dilution	number	number
		Extruded mixture of corn meal:bread crumbs, 50:50	Extruded mixture of corn meal:bread crumbs, 60:40
Salmonella spp	in 50 g	not detected	not detected
Coagulase positive *Staphylococci*	in 50 g	not detected	not detected
Sulphate-reducing *Clostridia*	in 1 g	not detected	not detected
Proteus varieties	in 50 g	not detected	not detected
Escherichia coli	in 50 g	not detected	not detected
Total number of yeasts	in 1 g	not detected	not detected
Total number of fungi	in 1 g	not detected	not detected
Total number of microorganisms	in 1 g	40	20

Obtained results indicate that the extrusion process has contributed to a complete reduction of microorganisms despite the relatively low extrusion temperatures (95-105 °C) and a very short extrusion time (6-10 s), but very high extrusion pressure, which ranges from 30 to 40 bar (Kormanjoš et al., 2007). In the extruded mixtures *Salmonella spp*., Coagulase positive *Staphylococci*, Sulphate-reducing *Clostridia*, *Proteus* species, *Escherichia coli*, were not detected and the total number of microorganisms is meeting the Regulations on the maximum quantities of hazardous substances and ingredients in animal feed (Službeni list SFRJ, 1990).

CONCLUSION

It was found that the main causes of high percentage of non used and withdrawn bread are:

1. bread is still a social category of food,
2. a relatively low price of bread,
3. a relatively low level of quality,
4. the habits of consumers to buy more bread than they consume,
5. food culture in which the stale bread is rarely used.

Extrusion process leads to improving the nutritive value of extruded corn meal mixture and bread crumbs due to increased content of total and reducing sugars, as well as, positive changes in the complex starch extrudates therefore increasing the digestibility of food, thereby increasing the performance efficiency. Heat treatment (temperature over 90 °C) along the extruder and the high pressure that occurs due to friction, contribute to reducing the total number of microorganisms in the product. Reduction of microorganisms provides a hygienic nutrient obtained. Extrusin of corn meal with bread crumbs may positively contribute to solving the problem of withdrawn bread in the bakery industry. The resulting extruded feeds are recommended in the nutrition of young animals, above all in feed production for fish and pets.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from the Ministry of Education and Science of the Republic of Serbia (Project III 46005).

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DETERMINATION OF NATURAL PHENOLS AFTER MICROWAVE-ASSISTED EXTRACTION

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ABSTRACT: Natural phenols are synthetized by large group of plants where they play an important role in plant metabolism and protection against pests. These compounds, in addition to being indispensable in plant metabolism, also exhibit complex biological activity in humans for what they are constantly in the focus of scientific interest. The aim of this study was to modify standard spectrophotometric method for polyphenols determination. Modification included application and optimization of microwave-assisted extraction which significantly reduced sample preparation time and improved extraction yields. Furthermore the goal of this study was to improve determination sensitivity by applying differential spectrophotometric measurement. Optimized analytical method was implied to determine anthocyanins, neutral and total polyphenols in different fruit and vegetable samples, as well as in cakes remaining after oil extraction of several oilseeds. Purification of extracts and fractionation of polyphenols to different classes was performed by using solid phase extraction. Individual acidic and neutral polyphenolic compounds were identified in plant extracts by thin layer chromatography.
Key words: *Natural phenols, microwave-assisted extraction, differential spectrophotometry, thin layer chromatography*

INTRODUCTION

Since ancient times it has been known that consumption of fruits and vegetables contributes to health and well being. Scientific development and improvement of analytical instruments enabled the identification of bioactive compounds responsible for positive health effects. Great number of published work describes antioxidant, anticarcinogenic and anti-inflammatory effects of polyphenols, as well as their positive effects on cardiovascular system (Higdon and Frei, 2003; Lazarou et al., 2007). For these reasons characterization of natural products in respect to the content of polyphenolic compounds and their identification, as well as improvement of existing analytical methodologies is of high scientific interest. In the analysis of natural phenols predominantly two techniques are used, spectrophotometric (Gonzalez et al, 2003) and chromatographic (Vinas et al., 2000), with chromatographic having advantages in respect to sensitivity, selectivity and reliability of determination, but, on the other hand, requiring much more expensive and sophisticated instrumentation and exploitation costs. Spectrophotometric methods allow determination without utilization of expensive and sophisticated analytical instrumentation that is often unavailable to some laboratories. The analyst should be aware that the application of these optical methods is limited by their sensitivity and that sample components interfere with spectrophotometric measurement. Determination of natural phenols in foodstuffs has been the subject of extensive research and many papers have been published. The aim of this work was to modify standard spectrophotometric method for phenol determination in natural products in order to reduce total analysis time and to improve sensitivity of determination.

MATERIALS AND METHODS

Instrumentation

Fruit, vegetable and oilseed cake samples were extracted by using modified domestic microwave oven (LG Electronics). Obtained extracts were evaporated to dryness by using vacuum evaporator (Roravapor R, Switzerland) and were fractioned on Baker Octadecyl SPE cartridges. Spectrophotometric measurements were made on a single-beam "Spekol" spectrophotometer.

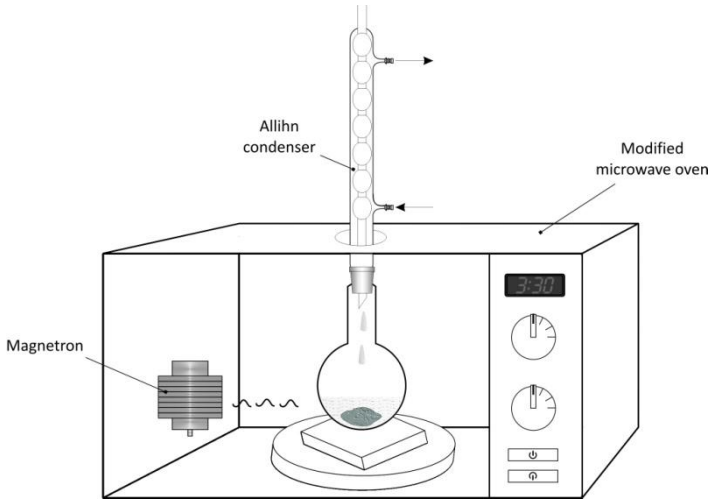

Figure 1. A modified domestic microwave oven used for microwave-assisted extraction.

Chemicals and Reagents

Solvents used for the extraction were prepared by mixing methanol, distilled water and acetic acid in ratio 14:5:1. Reagent of $AlCl_3$ for spectrophotometric measurement was obtained by dissolving 13.3 mg of $AlCl_3 \cdot 6H_2O$ and 40 mg of sodium acetate in distilled water and diluting to 100 ml. Standard rutin solution (1.02 mg/ml) was prepared by dissolving the glycoside in distilled water. For preparing acetate buffer (pH = 4.5) 60 g of acetic acid and 20 g of sodium hydroxide were dissolved in distilled water and diluted to 1000 ml.

All used chemicals were of p.a. purity grade and were supplied by following producers: Centrohem (methanol, ethanol, hydrochloric acid, sodium-carbonate, ethyl-acetate, formic acid), Zorka (acetic acid, sodium acetate), Lachner (sodium hydroxide), Kemika (alumina-chloride hexahydrate, buffer pH 1, benzene), Fluka (catechin, chlorogenic acid), Biochemica (Folin-Ciocalteau reagent, rutin three hydrate) and Macherey-Nagel (Silica gel G).

Sample Preparation

The content of different classes of polyphenols was determined in samples of pear, kohlrabi, cauliflower, aubergine and cakes of pumpkin seed and walnuts left after mechanical oil removal. Samples of fruits and vegetables were purchased at the local market and were prior extraction chopped and air dried at the room temperature. Oilseed cake samples were obtained from the Department of Food Technology, Faculty of Technology, University of Novi Sad. After drying all samples were grinded.

Samples (5 g) were transferred to the extraction cuvettes and 30 ml of extraction solvent was added. Microwave extraction followed the program: 1 minute heating at 160 W; 1 minute heating at 320 W; 2x5 minutes heating at 480W. Between all heating steps a short breaks (45 s) were made in order to avoid overheating. Obtained extracts were filtered and evaporated to dryness at 50°C applying a rotary vacuum evaporator. Dry residue was dissolved in 10 ml of 0.5 mol/l H_2SO_4 with ultrasound assistance. The extracts were further filtrated through HPLC 0.45 μm filters in order to prevent the channeling and pore blocking in subsequently applied SPE.

In order to remove sugars, aminoacids, proteins and other interfering substances, as well as to separate extracted compounds to different phenol classes, C18 SPE cartridges were used.

Fraction of Total Phenols
Cartridges were conditioned with 2 ml of methanol and 5 ml of 5 mmol/l H_2SO_4. The samples (2 ml) were passed through the column under the vacuum. After eluting the impurities with 2 ml of 5 mmol/l H_2SO_4 phenols were eluted with 2 ml of methanol and 5 ml of water.

Fraction of Neutral and Acidic Phenols
Neutral and acidic phenols were separated from the fraction of total phenols in which the pH was adjusted to 7 by adding 0.5 mol/l of NaOH and 0.05 mol/l of NaOH. For collecting the fraction the cartridges were previously conditioned with 8 ml of methanol and 4 ml of double distilled water. Previously obtained fraction of total phenols was passed through the column. Fraction of acidic phenolics was eluted with 10 ml of double distilled water and required further purification. Neutral polyphenols were stripped from the cartridge with 12 ml of methanol.

Fraction of Acidic Phenols
Eluted fraction of acidic phenols was further purified by SPE. Prior fraction introduction on SPE cartridge pH was adjusted to 2 with 2 mol/HCl. The cartridge was conditioned with 8 ml of methanol and 4 ml of 0.01 mol/l of HCl. Impurities were eluted by 5 ml of 0.01 mol/l HCl and acidic fraction was collected in 12 ml of methanol.

Determination of Total Polyphenols
Purified fraction of total phenols (3 ml) was mixed with 500 μl of Folin-Ciocalteau reagent, 2 ml of 20% of Na_2CO_3 and was diluted to 10 ml by distilled water. The blank and the standard solutions of chlorogenic acid were prepared in the same fashion. Absorbances were measured at 750 nm after allowing the reaction to proceed for 2h. By using calibration curve defined with standard solutions of chlorogenic acid the content of total phenols in the samples was expressed as chlorogenic acid equivalents (mg chlorogenic acid/g).

Determination of Neutral Polyphenols (Flavonoids)
Purified fraction of neutral polyphenols was evaporated to dryness and the dry residue was dissolved in 10 ml of 70% methanol. Dilute solution (5 ml) was mixed with 4 ml of $AlCl_3$ reagent. For defining calibration curve standard solutions of rutin were mixed with $AlCl_3$ reagent. Measured absorbances at 446 nm were very low due to low flavonoids content for what a differential spectrophotometry – the method for low absorbances was applied. In applied method 100% of transmission was set with pure solvent while 0% transmission was adjusted with the highest concentration of rutin standard solution. Flavonoid content was expressed as rutin equivalent (mg rutin/g).

Determination of Anthocyanins
Two probes of 1.5 ml of purified fractions of total phenols were transferred to volumetric flasks of 10 ml which were filled up to a mark with buffers of pH = 1 and pH = 4.5 for each probe. After allowing 15 minutes standstill the absorbances at 515 nm and 700 nm were measured. The concentration of total anthocyanins was expressed as cyanine-3-glucoside equivalent and was calculated according to:

$$C_{tot} = \frac{A_{tot} \cdot M}{\varepsilon \cdot l}$$

$$A_{tot} = (A_{515nm} - A_{700nm})_{pH1}$$

where:

A_{tot} was the total solution absorbance
$A_{515\,nm}$ was the solution absorbance at pH = 1 and 515 nm
$A_{700\,nm}$ was the solution absorbance at pH = 1 and 700 nm
M was the molecular mass of cyanine-3-glucoside (449.2 g/mol)
ε was the molar absorption coefficient of cyanine-3-glucoside
l was the optical path (10 mm)

The concentration of (C_{mon}) monomeric anthocyanins was expressed as cyanine-3-glucoside equivalent and was calculated according to:

$$C_{mon} = \frac{A_{mon} \cdot M}{\varepsilon \cdot l}$$

$$A_{mon} = (A_{515nm} - A_{700nm})_{pH1} - (A_{515nm} - A_{700nm})_{pH\,4.5}$$

where:
A_{mon} was the absorbance of total monomeric anthocyanins
$A_{515\,nm}$ was the solution absorbance at pH = 4.5 and 515 nm
$A_{700\,nm}$ as the solution absorbance at pH = 4.5 and 700 nm

The concentrations of total and monomeric anthocyanins were expressed as cyanine-3-glucoside equivalent (mg cyanine-3-glucoside/g) whereas the concentration of condensed anthocyanins was calculated from the difference of total and monomeric anthocyanins.

Thin Layer Chromatography
Plates for thin layer chromatography were prepared by applying a suspension of silica gel G on clean glass plates in 0.25 mm layer. Prior use plates were air-dried overnight and activated by drying in oven at 105°C for 1h.
All polyphenolic fractions together with available standards were simultaneously analyzed. The mixture of benzene:ethyl acetate:formic acid = 3:3:1 was used as a mobile phase. After chromatographic separation obtained chromatogram was visualized with the Folin-Ciocalteau reagent allowing 2h color to develop.

RESULTS AND DISCUSSION

The contents of flavonoids, anthocyanins (total, monomeric and condensed) and total phenolics, calculated by described methods are presented in Table 1 and are expressed as $x_{mean} \pm 2SD$, based on three separate sample analyses. Calibration curve defined with chlorogenic acid yielded a good linearity (y = 51.6x – 0.015; r = 0.9889) whereas, as expected on the basis of theoretical considerations of spectrophotometry, calibration curve for flavonoids, defined with rutin, produced non-linear dependence best approximated with logarithmic function (y = 0.123ln(x) – 0.284; r = 0. 9910).

Table 1. The contents of flavonoids, anthocyanins (total, monomeric and condensed) and total phenolics

Determined content					
Sample	Flavonoids[a]	Anthocyanins[b]			Total phenolics[c]
		Total	Monomeric	Condensed	
Pear	185.1[d]±0.9	4.2±0.2	2.1±0.3	2.1±0.2	431.8±0.9
Kohlrabi	185.9±0.8	33.8±0.4	nd	33.8±0.4	916.0±1.2
Cauliflower	202.6±0.8	15.1±0.2	5.0±0.4	10.1±0.3	1463.8±2.9
Aubergine	434.4±0.6	103.1±0.6	61.9±0.3	41.2±0.4	1593.8±1.6
Walnuts cake	35.6±0.4	0.8±0.2	0.4±0.2	0.4±0.3	186.4±0.6
Pumpkin seed cake	22.8±0.4	1.2±0.3	0.4±0.3	0.8±0.2	83.0±0.5

[a]*mg equivalent of rutin/100g dry weight*
[b]*mg cyanidine-3-glycoside/100g dry weight*
[c]*mg equivalent of chlorogenic acid/100g dry weight*
nd – not detected
[d]*mean value±2SD*

The sample of aubergine demonstrated to be the richest source of total phenols, flavonoids and anthocyanins, as known from the literature (Apak et al., 2007), and served as a reference sample. Available data on phenol content in other analysed samples were not so abundant in the literature.
Research conducted within this assay showed that flavonoids reacted with iron ions in alkaline media forming a dark blue product that produced very sensitive spectrophotometric response. On the basis of these observations it is anticipated to investigate more thoroughly this reaction and to compare the analytical performance with that of standard Markham method, in which the colored product is formed with Al(III).

Identification of Phenolic Compounds

Due to limited number of available standards and great diversity of natural phenolic compounds, only several compounds were reliable identified in natural extract (Table 2). Available standards that were analyzed simultaneously with prepared extract included as little as three standard compounds, more specifically rutin, catechin and chlorogenic acid. Even for fractions rich in phenolics, as determined by spectrophotometry, the assay demonstrated that applied chromatographic conditions were not adequate for separation and visualization of different phenolic classes requiring further optimization. For more reliable identification of natural phenols the use of more powerful instrumental analysis, such as HPLC MS is required.

Table 2. Qualitative analysis of different phenolic fractions of natural extracts by TLC

	Chromatogram	
Sample	Flavonoid fraction	Total phenolics fraction
Pear	-	One unidentified compound
Kohlrabi	One unidentified compound	One unidentified compound
Cauliflower	-	Catechin
Aubergine	-	Catechin Chlorogenic acid Two unidentified compounds
Walnuts cake	-	Catechin Two unidentified compounds
Pumpkin seed cake	-	Chlorogenic acid

By applying thin layer chromatography catechin was detected in samples of cauliflower, aubergine and walnuts cake, while chlorogenic acid was identified in aubergine extracts and pumpkin seed cake.

CONCLUSIONS

The content of total phenolics, total anthocyanins, monomeric and condensed anthocyanins, as well as flavonoid contents, were determined in different samples of fruit and vegetables as well as in two samples of oilseed cakes by applying differential spectrophotometric method and fractionation to different phenol classes by SPE. The extraction of all samples was performed in microwave field in open system. According to obtained results the highest content of total phenols and individual classes was observed in the sample of aubergine. Catechin was detected in samples of cauliflower, aubergine and walnuts cake, while chlorogenic acid was identified in aubergine extracts and pumpkin seed cake.

ACKNOWLEDGEMENTS

This paper is a result of bilateral cooperation between Republic of Serbia and Kingdom of Spain, supported by the Ministry of Education and Science of the Republic of Serbia and Spanish Ministry of Education and Science (PRI-AIBSE-2011-1228).

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EFFECT OF COMPOSITION ON BIOACTIVE PROFILE OF CHOCOLATE LIQUEURS

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ABSTRACT: Chocolate liqueurs are attractive beverages because of their desirable sensory properties, but they could also be presented as a good source of bioactive compounds, derived from cocoa. Cocoa and cocoa products have received increased scientific attention due to their health benefits attributable to interesting phytochemical composition, especially high content of polyphenols and methylxanthines. Since the processing parameters and different recipes, in chocolate liqueur production, can influence the content of bioactive compounds in the final product, the purpose of this study was to determine the best combination of the type of chocolate (regarding cocoa solid content) and ethanol content aimed to produce functional chocolate liqueurs. UV/VIS spectrophotometric methods were applied in order to compare the composition of polyphenols between dark chocolate liqueurs and milk chocolate liqueurs. Antioxidant capacity of prepared liqueurs was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) assays. Among the studied samples, dark chocolate liqueur containing 20% of ethanol and milk chocolate liqueur containing 30% of ethanol were presented as the richest sources of bioactive compounds with strong antioxidant properties.
Key words: *bioactive compounds, chocolate liqueur, cocoa, ethanol*

INTRODUCTION

Alcoholic beverages comprise a large group of products that contain varying amounts of alcohol (ethanol) which, produced on an industrial scale, include beer, wine and distilled spirits such as brandy, whisky, rum, gin, cognac, vodka, tequila and liqueurs (Cacho and Lopez, 2005). Liqueurs are popular alcoholic beverages made by direct extraction of fruits, nuts or herbs with spirits (Wende and Beta, 2010). Chocolate liqueurs are made of liqueur base, chocolate and alcohol (with minimum volume of 15%), without distilling process. In the last few years, cocoa products have received much attention due to the natural composition of cocoa, which contains a mixture of bioactive compounds, with polyphenols as predominant. Regarding their significant content, which enables them to exhibit different levels of antioxidant activities, it is possible to postulate that there may be direct or indirect synergysm between bioactive compounds in cocoa in delivering their health benefitial properties due to their significant polyphenol content from which they may possess different levels of antioxidant activity (Jalil and Ismail, 2008). Besides polyphenols, cocoa contains substantial content of minerals, fats, proteins and caffeine that can provide significant contribution to consumers' nutrient intake, as well as to unique sensory characteristics of cocoa based products (Wilson and Temple, 2004). Antioxidants in alcoholic beverages, such as whisky, liqueurs, beer and red wine, are considered to have protective effects on ethanol-induced health damage (Wende and Beta, 2010). In a study by Blanco-Colio et al. (2007) it was demonstrated that consumption of some alcoholic beverages can be associated with a reduction of cardiovascular mortality, an effect partly attributed to their antioxidant properties. Since there is no distilling process, which can significantly reduce antioxidant content, chocolate liqueurs are characterized with higher content of native bioactive compounds. Favorable and stable aroma plays an important role in consumer's acceptance and contributes to the enjoyment of chocolate alcoholic beverages (Wende and Beta, 2010).

Sensory properties of liqueurs are strongly associated with their phenolic content (Montoro et al., 2006), which contribute to their astringent but pleasant taste (Stampar et al., 2006). The presence of other nutrients like proteins, carbohydrates and fat in chocolate liqueurs, and the interaction between them, may directly or indirectly affect the bioavailability of polyphenols (Jalil and Ismail, 2008). Type of chocolate used for chocolate liqueur production, as well as the content of alcohol, are important factors that influence antioxidant properties of the final liqueur product. Therefore, this study was conducted to investigate the effect of two main ingredients (chocolate and alcohol) and their combination in order to develop functional chocolate liqueur.

MATERIAL AND METHODS

Preparation of samples

Two types of chocolate (dark chocolate with 50% of cocoa solids (DC) and milk chocolate with 32% of cocoa solids (MC)) were combined with 3,3% cream liqueur base and spirits containing 20%, 25% and 30% (v/v) of ethanol in order to prepare chocolate liqueurs. Chocolate was added in a content of 10% at temperature of 30 ^{0}C. Samples were well homogenized and left to stand for one week.

Chemicals

Folin–Ciocalteu, sodium carbonate, formaldehyde, methanol, n-butanol, acetone and hydrochloric acid were supplied by Kemika (Zagreb, Croatia). DPPH (2,2-diphenyl-1-picrylhydrazyl) was supplied by Fluka (Buchs, Switzerland) . Vanillin, 4-dimethylaminocinnamaldehyde, Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ABTS (2,20-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt) and gallic acid (GA) were obtained from Sigma–Aldrich (Steinheim, Germany).

Determination of total phenol (TPC) and flavonoid content (TFC)

Total phenol and flavonoid content of the samples were determined spectrophotometrically according to a modified method of Lachman et al. (1998). Flavonoid content was calculated as subtraction of total phenol and non-flavonoid content. Gallic acid was used as the standard and the results were expressed as mg gallic acid equivalents (GAE) per litre of liqueur sample (Kramling & Singleton, 1969). All measurments were performed in duplicate.

Determination of flavan-3-ol content

Vanillin assay

Samples were analyzed using a method described by Di Stefano et al. (1989). The content of flavan-3-ols was calculated according to the formula: (+)-catechin = 290.8 ×ΔE, and the results were expressed as mg (+)-catechin/L of liqueur sample.

Reaction of 4-dimethylaminocinnamaldehyde (p-DAC)

A standard procedure reported by Di Stefano et al. (1989) was used. The content of flavan-3-ols was calculated according to the formula: (+)-catechin = 32.1 ×ΔE, where ΔE is the subtraction of absorbances between the tested liqueur sample and appropriate blanks. The results were expressed as mg (+)-catechin/L of the liqueur sample.

Quantative determination of proanthocyanidins

Proanthocyanidins were analyzed by the procedure described by Porter et al. (1986), with some modifications. The quantity of proanthocyanidins was determined from a standard curve of cyanidin chloride treated with BuOH–HCl–Fe^{III} mixture, and expressed as mg cyanidin chloride equivalents (CyCl)/L of liqueur sample.

Determination of antioxidant activity

DPPH radical scavenging assay
Antioxidant capacity of the liqueur samples was determined using the DPPH radical scavenging assay described by Brand-Williams et al. (1995). Antioxidant capacity was expressed as mmol/L Trolox equivalents.

ABTS radical scavenging assay
The Trolox equivalent antioxidant capacity (TEAC) of liqueur samples was also estimated by the ABTS radical cation decolorization assay (Re et al., 1999). The results, obtained from duplicate analyses, were expressed as Trolox equivalents and derived from calibration curve determined for this standard (100-100 µM).

RESULTS AND DISCUSSION

Figure 1 shows total phenol content (TPC) of the liqueur samples expressed as mg GAE/L. TPC of dark chocolate liqueurs (DC) ranged from 613,63 mg GAE/L to 704,54 mg GAE/L, while in milk chocolate liqueurs (MC) ranged from 372,72 mg GAE/L to 418,18 mg GAE/L.

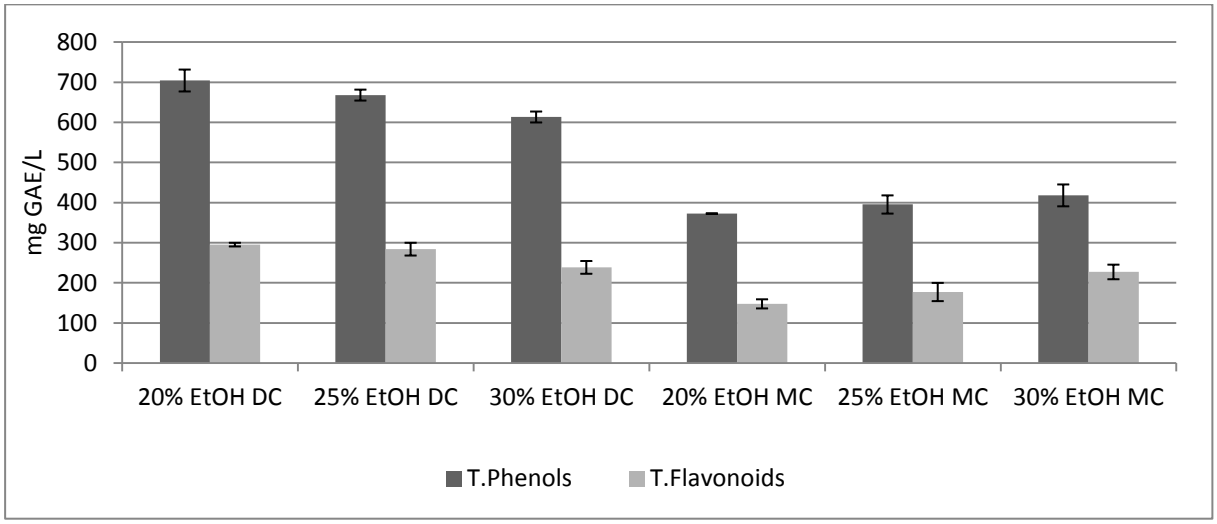

Fig.1. The content of total phenols (TPC) and flavonoids (TFC) in chocolate liqueurs

These results are consistent with the previously published results of Arts et al. (1999) who concluded that polyphenol content of chocolate was increased proportionally to the content of cocoa solids. The highest value of TPC among dark chocolate liqueurs was obtained in the sample containing 20% of ethanol, while the sample with 30% of ethanol was characterized with the lowest TPC which indicates that higher percent of ethanol exhibited lower content of phenol compounds originally present in the chocolate. In opposite to dark chocolate liqueurs, extractability of phenolic compounds in milk chocolate liqueurs was increased with higher ethanol content in the samples. Total flavonoid content (TFC) followed the same pattern of results as total phenol content (Figure 1). The highest TFC value (295,45 mg GAE/L) was obtained in dark chocolate liqueur containing 20% of ethanol, while among milk chocolate liqueurs the highest TFC value (227,27 mg GAE/L) was determined in the liqueur with 30% of ethanol.

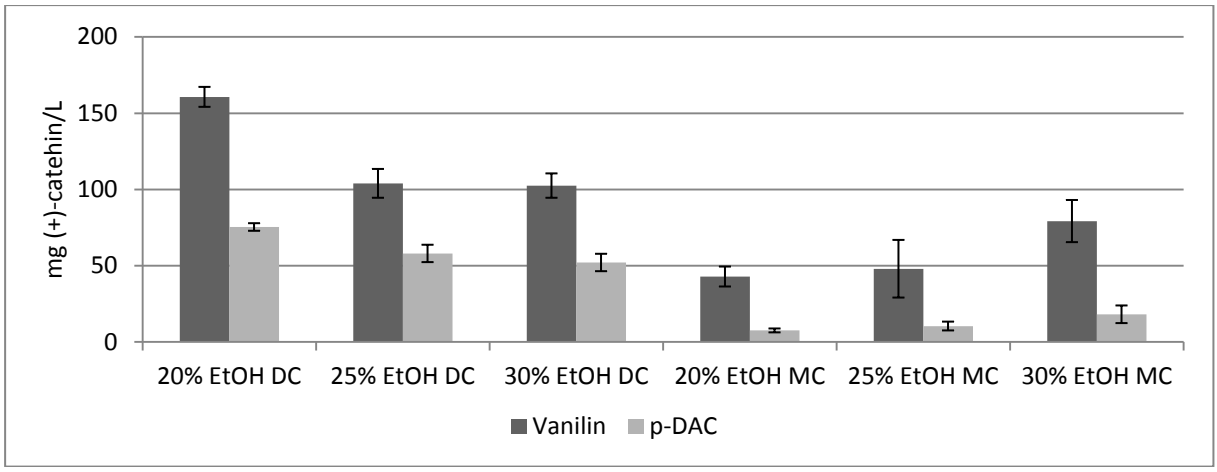

Fig. 2. The content of flavan-3-ols in chocolate liqueurs determined by vanillin and *p*-DAC assays

Flavan-3-ol content of the samples was determined by two assays, in reaction with vanillin reagent and with *p*-dimethiylaminocinnamaldehyde (*p*-DAC), which is shown in Figure 2. Regarding the type of chocolate, content of flavan-3-ols was higher in dark chocolate liqueurs than in samples with milk chocolate. Considering the ethanol percentage, the highest value of flavan-3-ols was expressed in samples with 20% of ethanol, in dark chocolate liqueurs, and in samples with 30% of ethanol, in milk chocolate liqueurs. Since the principle of these two assays is based on the reaction of *p*-DAC reagent with hydroxyl groups in specific C-atom positions in the flavan-3-ol molecules, the reaction of *p*-DAC reagent usually exhibits lower numeric values for the content of flavan-3-ols in comparison with the vanillin assay.

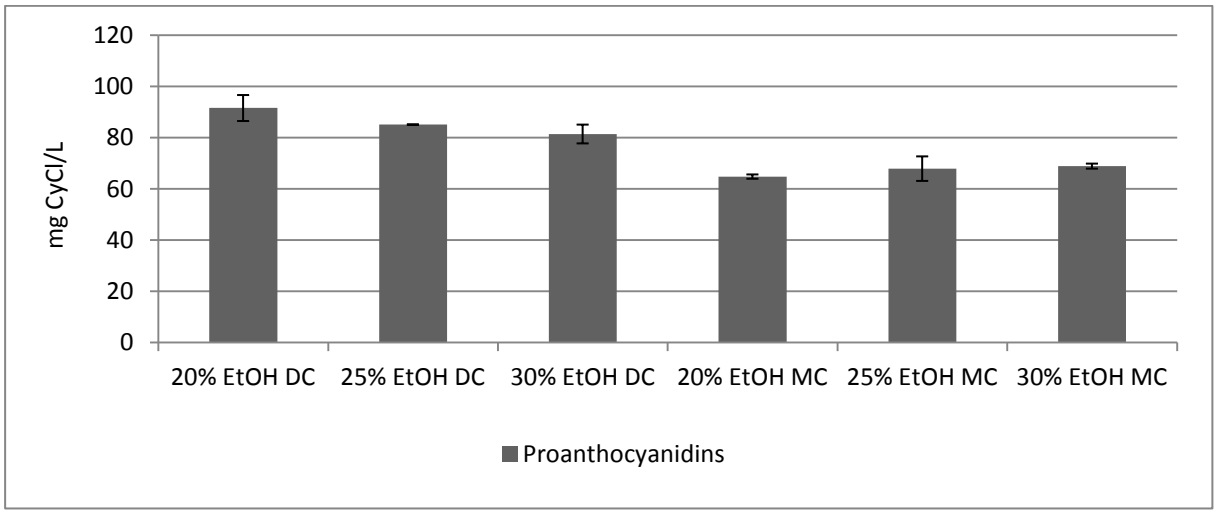

Fig. 3. The content of proanthocyanidins in chocolate liqueurs

The content of proanthocyanidins in chocolate liqueurs exhibited the same trend as polyphenols, observed as an increase of extracted compounds as the content of ethanol was also increasing, in the milk chocolate liqueurs, and decreasing in the case of dark chocolate liqueurs. It is well established that oligomeric cocoa proanthocyanidins exhibit higher antioxidant capacity than monomeric catechins, which is attributed to higher polymerization degree of proanthocyanidin molecules. Therefore, the content of proanthocyanidins should be regarded as an important paremeter for optimization of processing parameters in the production of raw materials for chocolate liqueur preparation, implying that their production should be performed in a way to preserve as highest content of proanthocyanidins as possible. According to the obtained results (Figure 3), the highest proanthocyanidin content

was determined in dark chocolate liqueur with 20% of ethanol (91,54 mg CyCl/L) and among milk chocolate liqueurs, the highest content of proanthocyanidins was observed in liqueur containing 30% of ethanol (68,87 mg CyCl/L). It has been known that proanthocyanidins form complexes with proteins and carbohydrates (Davis & Hoseney, 1979; Leinmuller et al., 1991) so it's likely that proteins in milk chocolate are the reason for the lower content of proanthocyanidins in milk chocolate liqueurs in comparison with dark chocolate liqueurs.

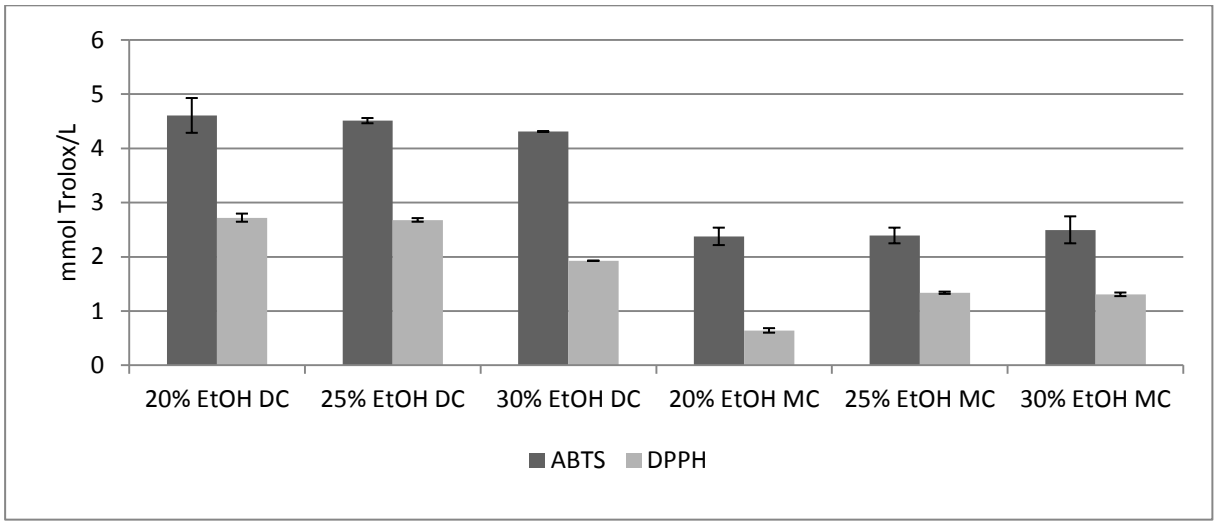

Fig. 4. Antioxidant activity of chocolate liqueurs determined by ABTS and DPPH assays

Previous studies have shown that phenols and flavonoids have antioxidant or antiradical activities (Montoro et al., 2006). As shown in Figure 4., antioxidant activity determined by ABTS and DPPH assays correlated with the result pattern from polyphenol analysis. Antioxidant activity of dark chocolate liqueurs was reduced with higher ethanol content while in milk chocolate liqueurs the influence of ethanol was opposite. Some studies indicate that antioxidant properties of alcoholic beverages are influenced by many factors, such as their phenolic content, time and method of ageing and addition of flavour and colour additives. (Bartoszek and Polak, 2011). Additionally, Tabernero et al. (2006) pointed out that antioxidant capacity of milk containing products could be modified by the presence of proteins from the milk, which was also observed in the present study. This effect could be explained by the formation of milk protein-polyphenol complexes which finally reduce antioxidant capacity but the mechanism of these interactions is still unclear and requests further investigations.

CONCLUSIONS

Among chocolates containing different percentage of cocoa solids and spirits containing different percentage of ethanol, used in production of cocoa based alcoholic beverages, dark chocolate liqueurs containing 20% of ethanol and milk chocolate liqueurs containing 30% of ethanol have the best potential to be considered as functional beverages, which may have positive effect on human health. To determine other positive effects of these products as well as stability of their bioactive composition further studies are required.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science, Education and Sports, Republic of Croatia project 058 3470. The authors are grateful to Croatian food industry Zvečevo d.d. for supplying the samples used in the study.

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EFFECT OF SOYA MILK ON ANTIOXIDANT ACTIVITY CHANGES IN CHOCOLATE

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ABSTRACT: Properties of chocolate depend on raw chocolate composition, manufacturing process and properly guided phase of precrystallization. In this paper chocolate was produced in a non-conventional way, in the ball mill. Milk powder, as most commonly used raw material for milk chocolate production, was replaced with soy milk powder. In Western countries, soya milk is used in a diet of people who are intolerant to lactose, or who do not consume foods of animal origin. Three types of chocolate have been produced: R1 chocolate mass with 20% cow milk powder, chocolate mass R2 with 15% soy milk powder and R3 chocolate mass with 20% soy milk powder in laboratory ball mill.
The aim of this research was to examine the influence of the milk powder and soya milk powder (15% and 20%) on the total phenolic content. The total phenolic content (TPC) increased with the increase of soya milk content in chocolate samples. Antiradical scavenging activity was in a direct relationship with the total phenolics content. The lowest IC_{50} showed the sample of chocolate with 20% of soya milk.
Key words: *chocolate, soya milk, ball mill, solid fat content, hardness, total phenolics, antioxidant activity*

INTRODUCTION

The production of chocolate in the ball mill has been studied in the last ten years (Lucisano et al., 2006; Alamprese et al., 2007). The ball mill replaces the standard two stage process of chocolate manufacturing: refining and conching occur at the same time. Depending on the time of grinding, ball size and weight of balls, we get the optimal particle size distribution as it is obtained by standard procedure of production. By grinding chocolate in the ball mill or the five-roll refiner, we get the optimal particle size distribution (Afoakwa et al. 2007, Afoakwa et al. 2008). By grinding and continuous recirculation of chocolate mass at a particular temperature we get a stable suspension of solid particles in cocoa butter (Prawira & Barringer, 2009). Melted chocolate is a complex rheological system where the solid particles are dispersed in the fat phase. The fat phase consists of both cocoa butter and milky fat (or soya oil from soya milk). The solid phase is not uniformly dispersed phase, because the particles are with different distributions in size, shape and properties on the surface. The influence of grinding on chocolate mass is reflected in the gradual reduction of the shear stress due to which it gets the appearance of a homological suspension that starts to flow. The stable chocolate mass is tempered before moulding with the aim of creating crystallizing centers in the stable crystal form V cocoa butter (Pajin & Jovanovic 2005, Garti & Sato 1988, Timms 2003). So formed crystals allow moulding and solidification of chocolate i.e. the best possible physical of chocolate.
The chocolates are produced with cow milk in powder and soya milk in powder. Soy flour has great potential for replacing milk powder in chocolate due to its high protein content (Akinwale 2000). Unlike the cow milk, soya milk has a reduced content of polyunsaturated fatty acids and does not contain lactose and cholesteroll. The energetic value of 100 g of whole milk is 64 Kcal, while the soya milk has 49 calories.

Health benefits of various cocoa products arise from cocoa polyphenols and their antioxidant capacity. Recent studies show that soya contains a wide range of bioactive components which are proved to be very effective in preventing heart disease, blood vessels and cancer, which successfully classifies this raw material among functional foods, or food with positive effect on human health (Kurzer 2003, Sacks et al. 2006).
Soya contains flavonoids, isoflavonoids, isoflavones and other polyphenols. These compounds may be important in reducing risk of atherosclerosis, as they have been shown to have favorably effect on plasma lipids and inhibit platelet aggregation (Sacks et al. 2006).
Epidemiologic data support the idea that health benefits are linked to the consumption of certain flavonoids. Flavanol-rich, plant derived foods and beverages include various fruits, wine, tea, as well as cocoa and cocoa products – chocolate (Keen 2001). A recent study suggested that milk proteins reduce the antioxidant activity of milk chocolate, compared with dark chocolate (Serafini et al. 2003).
This paper reports the development of soya-enriched chocolate and characterization of these products. Analyzed is improvement in terms of a total polyphenols and antioxidant activity caused by substituting milk powder with soya milk.

MATERIALS AND METHODS

Materials

The raw materials used for making milk chocolate with soya milk are: cocoa butter (Theobroma, Amsterdam), cocoa liquor (Cargill), medium-grain sugar (Crvenka AD, Serbia), whole cow milk powder (total fat 25%, proteins 28% and carbohydrates 37%) - Imlek, Serbia, skimmed milk powder (total fat 1%, proteins 35% and carbohydrates 51 %) - Imlek, Serbia, soya milk powder (total fat 26%, proteins 44% and carbohydrates 18.5 %) - Olvebra Provesol PSA, Brazil, hazelnut paste (total fat 25%, proteins 28% and carbohydrates 37%) - Arslanturk, Turska, Ethylvanilin (FCC, Norway), Soy lecithin with a minimum insoluble content of 65% in acetone (Soyaprotein AD, Serbia), Polyglycerol polyricinoleate or PGPR (Danisco, Malaysia).

Methods

The description of experiment:
The chocolate was manufactured in a laboratory ball mill with a homogenizer (capacity 5kg), domestic manufacturer. All raw materials were added to homogenizer exept for the 10% cacao butter. The mixing time was 20 minutes. After mixing the mass was transfered to the ball mill. The milling time was 90 minutes and the remaining quantity of the cacao butter was added in the 80th minute.The diametar of the balls in the mill is 9.1mm and the rotation speed of mixer is 50rpm.The ball mill is equipped with the mass-circulation system, the speed of which is 10 kg/hour. The internal diameter of ball mill is 0.250 m, and the height is 0.31 m. The volume of space provided for balls and 5 kg of chocolate mass is 0.0152 m^3.
Three types of chocolate mass were produced (Table1): R1(the chocolate mass with 20% cow milk powder), R2 (chocolate mass with 15 % of soya milk powder), R3 (chocolate mass with 20% of soya milk powder).

Table 1. Composition of chocolate mass

Ingredients	R1	R2	R3
		Content (%)	
Cocoa liquor	10.3	10.3	10.3
Sugar	41.5	50.3	47.2
Cocoa butter	19.8	19.8	19.8
Whole cow milk powder	20.0	0.0	0.0
Skimmed cow milk powder	5.7	0.0	0.0
Soya milk powder	0.0	15.0	20.0
Hazelnut paste	2.1	4.0	2.1
Soya lecithin	0.3	0.3	0.3
PGPR	0.2	0.2	0.2

The precrystallization of chocolate masses was performed in laboratory-modified Brabender precrystallization pharinograph. The temperature of precrystallization was 26, 28^{0}C and 30^{0}C. The flow od precrystallization is monitored indirectly through changes in internal resistance on the occasion of mixing, which is registered on force/time diagram. Precrystallized chocolate masses are cast in moulds and cooled at a temperature of 10-14°C (Pajin 2009). Symbols of used chocolate masses are given in the Table 2 .

Table 2. Symbols of chocolate mass

Symbol	Types of chocolate mass	Refinig time (min)	Temperature of precrystallization (°C)
R1-90-26	R1	90	26
R1-90-28	R1	90	28
R1-90-30	R1	90	30
R2-90-26	R2	90	26
R2-90-28	R2	90	28
R2-90-30	R2	90	30
R3-90-26	R3	90	26
R3-90-28	R3	90	28
R3-90-30	R3	90	30

Sample preparation for determination of total phenolic content and antioxidant activity:
In order to eliminate lipids from samples of chocolates 2.0 g of each sample was extracted three times with 10 ml hexane. The defatted solids were air-dried during 24h to remove the residual organic solvent. The phenolic compounds were extracted twice from 2 g of defatted chocolate products with 5 ml of aqueous methanol (70%) for 30 min in an ultrasonic bath (Model USK 28, power 600W, frequency 40 kHz, EI Nis, Serbia). After each extraction, the mixture was centrifuged for 10 min at 3000 rpm and supernatant was decanted.
Determination of total phenolics content:
The content of total phenolics in chocolate was determined by modified Folin/Ciocalteu method (*Singleton & Rossi 1965*) 100µl of extract was shaken for 1 min with 500 µl of Folin/Ciocalteu reagent and 6 ml of distilled water. After the mixture was shaken 2 ml of 15% Na_2CO_3 were added and the mixture was shaken once again for 5 min. The solution was brought up to 10 ml by adding distilling water. After 2h, the absorbance was measured on the UV/visible spectrophotometer (Ultraspec 3300 pro, Amersham Bioscience, Sweden) at 750 nm. The TPC was assessed by plotting the gallic acid calibration curve and expressed as milligrams of GAE per gram of dried sample. All tests were carried out in triplicates.
Determination of DPPH radical scavenging activity:
Antioxidant activity of three chocolate samples was measured on the basis of scavenging activities of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Cavin *et al.* 1998). In 50 µl of samples of chocolate of various concentrations were added 3.95 ml of methanol and 1 ml 0.2 mM of DPPH methanol solution. After 30 min of incubation in the dark at room

temperature, the absorbance was measured against a blank at 517nm using UV/visible spectrophotometer (Ultraspec 3300 pro, Amersham Bioscience, Sweden). Inhibition of DDPH radical was calculated as a percentage (%) using formula:
Percentage inhibition (%) = [(A control –A sample) /A control] x 100
where A control is the absorbance of control reaction and A sample is absorbance of test compounds. IC50 values were calculated from the regression equation, prepared from the concentration of the samples and percentage inhibition of free radical formation. All tests were carried out in triplicates.

DISCUSSION AND RESULTS

The antioxidant activities and total phenolics of three samples of chocolate are shown in Table 3.
The results of this study imply that chocolate (as cocoa product) is important source of dietary antioxidants. According to the literature data, total phenolic content varies greatly depending on the solvent and procedure for the extraction of polyphenols. The results presented in this study are in accordance with Waterhouse, Shirley and Donovan (1996).

Table 3. The antioxidant activities and total phenols in samples of chocolates

Sample	**R1**	**R2**	**R3**
TPC (mg GAE /g sample)	$6.45 \pm 0{,}06$	$7.77 \pm 0{,}04$	$8.26 \pm 0{,}07$
DPPH (IC_{50}) (µg/ml)	57.7	56.4	52.5

They have found 5.0 mg polyphenols/g milk chocolate using the colorimetric Folin/Ciocalteu method. The results show that by replacing the cow milk with soya milk the total content of polyphenols rises (20.5 and 28.1% respectively) and these chocolates can be relevant source of phenolic antioxidants. Also, our study indicated that satisfactory scavenging ability on DPPH radicals could be the result of phenolic compounds in sample of chocolate with higher content of soya milk. IC50 were lower for sample R2 and than R3 for 2.3% and 9% respectively.

CONCLUSION

Changes in the composition of raw material i.e. replacement of cow milk powder by soya milk powder result in the increasing content of polyphenols. The increase of total polyphenols in the chocolate mass R3 was by 28.1 % comparing to the R1, and in the chocolate mass R2 by 20.5% comparing to R1.

ACKNOWLEDGEMENTS

This work funded by the Serbian Ministry of Science and Technological Development (Project TR 31014).

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BIOACTIVE COMPOUNDS CONTENT, CYTOTOXIC AND ANTIOXIDANT/PROOXIDANT ACTIVITY OF DIFFERENT COCOA PRODUCTS ON HUMAN LARYNGEAL CARCINOMA CELL LINE

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ABSTRACT: Cocoa and their products contain methylxanthines, peptides, biogenic amines and micronutrients which can influence the beneficial health effects of cocoa products, by either enhancing or reducing their biological activity. In this study the relation of polyphenols, methylxanthines and minor and major elements of several cocoa products with their cytotoxic activity and reactive oxygen species formation on one human carcinoma cell line was determined. Non-fat cocoa solids (NFCS), total polyphenol content (TPC) and methylxanthines content of cocoa product extracts were determined using UV/Vis spectrophotometric methods and HPLC analysis. Inductively coupled plasma atomic emission spectrometry (ICP-AES) was used for the determination of major and minor elements present in different cocoa products. Cytotoxic and anti-oxidative/prooxidative effects of cocoa product extracts in the presence of hydrogen peroxide were determined on human laryngeal carcinoma cell line (HEp2). The cocoa products containing higher NFCS exhibited higher TPC and methylxanthines content, while the content of major and minor elements was characterized by higher variability depending on the cocoa product. Experiments revealed a strong relationship between the type of product/concentration/time of exposure and antioxidant/prooxidant character of cocoa products. The observed prooxidant activity of dark chocolate and cocoa liquor may be attributed to higher contents of methylxanthines and transition metals present in these products.
Key words: *antioxidant activity, cocoa, cytotoxicity, non-fat cocoa solids, polyphenols, methylxanthines, minerals*

INTRODUCTION

Considerable experimental evidence has supported a key role of reactive oxygen species (ROS) as an important factor in the development of numerous diseases caused by oxidative damage (Yokozawa et al., 2007). The intake of naturally occurring nutritional sources of antioxidants, such as fruits, vegetables, tea, cocoa or wine, may attenuate the damage caused by oxidative challenges. Polyphenolic compounds, abundant in these nutritional sources, could play a major role in enhancing the antioxidant system, since they behave as ROS scavengers, metal chelators and enzyme modulators (Pietta et al., 1998). Cocoa products are studied for the most part because of the *in vitro* antioxidant and antiradical properties of their bioactive constituents on different cellular models of oxidative stress. The content of cocoa polyphenols is especially important, due to a great number of studies which have reported the health benefits of these bioactive compounds. It has been previously established that cocoa extracts containing polyphenolic compounds have the ability to scavenge ROS and protect human HepG2 liver cells (Martìn et al., 2008), colonic cancer cells (Caco-2 cells) (Carnésecchi et al., 2002) and rat adrenal pheochromocytoma cells (PC12 cells) (Cho et al., 2008) against oxidative stress. However, these effects cannot be attributed solely to polyphenols, given that cocoa and their products also contain methylxanthines, peptides, biogenic amines and micronutrients which can influence the

observed beneficial health effects of cocoa products, by either enhancing or reducing their biological activity (Visioli et al., 2009; Abbe Maleyki and Amin, 2008).
Cocoa derived products are the favourite food items of consumers of all age, among which children are the most susceptible age group to nutrients intake from such type of food items. In view of the complex matrixes of cocoa products having high sugar and organic contents, it is of great importance to precisely determine the bioactive content of this group of food products and to reveal their biological activity in different stress subjected conditions. Literature data regarding the effect of bioactive compounds in commercial types of cocoa products and their biological activity on cell lines are lacking, which indicates the necessity for analysis of these frequently consumed products. Moreover, the biological activities of various cocoa products have been determined mainly using individual polyphenolic compounds, such as procyanindin fractions or cocoa powder extracts, while studies using cocoa extracts of usually consumed cocoa products, especially chocolates are scarce. Therefore the present study reports the concentrations of polyphenols, methylxanthines and minor and major elements of several cocoa products and relates them with their cytotoxic activity and ROS formation on one human carcinoma cell line.

MATERIAL AND METHODS

Four different cocoa products (cocoa liquor, dark, semisweet and milk chocolate) were supplied by the leading Croatian chocolate manufacturer (Zvečevo food industry, Požega Croatia). The NFCS content of cocoa products was determined using a well established procedure within the confectionery industry (Balimann et al., 1999), based on the use of theobromine as a marker of NFCS.

Determination of polyphenols and methylxanthines content

The cocoa samples were prepared, extracted and analyzed for phenolic compounds and methylxanthines according to the procedures described by Belščak et al. (2009). Total phenol content (TPC) was determined spectrophotometrically according to a modified method of Lachman et al. (1998), and the results were expressed as mg gallic acid equivalents (GAE)/g of cocoa product. The content of methylxanthines, theobromine and caffeine was determined using a high performance liquid chromatography method developed by Belščak-Cvitanović et al. (2011).

Mineral analysis of cocoa products

Approximately 0.15 g of cocoa product was weighted into Teflon reaction vessels (in triplicate), and 5 mL of nitric acid (50:50 v/v) with 2 mL H_2O_2 was added to each sample. For the microwave assisted digestion a MWS-2 Microwave System Speedware BERGHOF was used, and the digestion procedure was conducted in three steps for 15 minutes each, at T(°C) and total power (W): 1.–110°C/60W, 2.–170°C/75W and 3.–140°C/50W. The resulting clear solutions of digestion procedure were then brought to 10 mL with ultrapure water. Mineral content of cocoa products was determined with a Prodigy High Dispersive ICP spectrometer (Teledyne Leeman, Hudson, NH, USA) working in a simultaneous mode. The instrument operated with optimal parameters: a radiofrequency power of 1.1 kW; plasma gas flow rate (Ar) of 18 L/min and auxillary gas flow rate (Ar) of 0.8 L/min. All standard deviations were based on measurements in triplicate and amounted to less than 10%.

Determination of biological activity *in vitro*

In order to determine the biological effects of cocoa product extracts, the previously prepared extract was evaporated to dryness and re-dissolved in sterile water. The cocoa products extracts were prepared with different concentrations (ranging from 0.5 x to 2.5x) in growth medium (DMEM). Cytotoxicity of cocoa products was determined by neutral red (NR) assay, while reactive oxygen species formation in the cells after the treatment with cocoa product extracts was determined by dichlorohydrofluorescein assay using microplate reader as described by Durgo et al. (2011). Also, antioxidative potential of cocoa product extracts was

determined prior to treatment with cocoa product extracts, on cells treated with 250 μM hydrogen peroxide for half an hour. Decrease of ROS formation in the cells pre-treated with cocoa extracts in comparison to positive control (250 μM hydrogen peroxide only) would indicate its antioxidative nature.

RESULTS AND DISCUSSION

Quantitative analysis of bioactive compounds of cocoa products (table 1) revealed a correspondence between the increase of NFCS and polyphenolic compounds, as well as methylxanthines of each cocoa product. Based on these results, the higher amount of NFCS indicates the higher phenolic and methylxanthines content of a cocoa product, which has been previously established (Miller et al., 2009; Belščak et al., 2009). However this does not apply to mineral content of cocoa products. The contents of potassium, calcium, sodium and aluminium are not affected by the NFCS content, which indicates that other ingredients of cocoa products may be the source of these major elements. As it can be seen from the results, magnesium and potassium are the prevalent minerals of cocoa products, while the contents of Cd, Co, and Pb are below the limits of detection (1.39, 1.19, and 1.13 μg/g, respectively). Nickel is found in a concentration range 0.10-0.60 μg/g. Similar concentrations are previously reported by Dahiya et al. (2005) in 69 different brands of chocolates and candies mainly made from cocoa, milk solids, dry fruits, fruit flavours and sugar.

Table 1. Polyphenolic and methylxanthines content of water extracts of cocoa products and the content of major and minor elements of cocoa products

	Milk chocolate	**Semisweet chocolate**	**Dark chocolate**	**Cocoa liquor**
NFCS (%)	5.07 ± 0.36	14.20 ± 1.01	23.60 ± 1.84	55.91 ± 0.56
TPC (mg GAE/g)	1.34 ± 0.02	1.65 ± 0.08	1.82 ± 0.05	2.31 ± 0.01
Methylxanthines* (mg/g)	2.38± 0.27	3.75 ± 0.14	4.50 ± 0.36	5.90 ± 0.22
Major elements (mg/g)				
K	1.29	0.76	2.99	0.55
Mg	0.53	0.84	1.54	2.71
Ca	1.43	0.37	0.59	0.84
Na	0.55	0.12	0.08	0.21
Al	0.20	0.04	0.05	0.08
Minor elements (μg/g)				
Fe	1.93	3.35	4.97	8.17
Zn	1.96	1.85	3.64	5.61
Cr	1.63	1.54	2.52	4.69
Cu	0.62	1.02	1.73	3.28
Mn	0.49	1.05	1.48	2.76
Ba	0.41	0.55	0.51	1.11
Sr	0.14	0.25	0.42	0.88
Ni	0.10	0.18	0.33	0.60

*TPC – total phenol content, *- Σ theobromine and caffeine*

None of the examined extracts caused 50% of cell death (figure 1). Cocoa liquor caused dose-response decrease in cell survival during 1 hour of incubation and prolonged incubation (2h). Dark chocolate decreased the cell survival in dependence of concentration and time of exposure, while semisweet chocolate showed its cytotoxic effect after prolonged exposure of HEp2 cells (2h). Carnésecchi et al. (2002) observed that the flavanol and procyanidin contents of the cocoa samples are directly related to their anti-proliferative effect on the human colon cancer cell line Caco-2. According to their results, cocoa powder extract caused only 25% of growth inhibition, whereas procyanidin enriched cocoa extract caused a

75% growth inhibition of Caco-2 cells. Since the results of our study revealed that milk chocolate containing the lowest NFCS had no effect on HEp2 cells survival, while the highest growth inhibition (27%) of cells was induced by cocoa liquor, after 2 hours of treatment, a correlation between the cytotoxic activity and NFCS content of cocoa products has been confirmed.

Since reactive oxygen species are formed continuously in cells as a consequence of both, oxidative biochemical reactions and external factors, the cells were also treated with 250 μM hydrogen peroxide for 30 min, prior to treatment with cocoa product extract. According to our results, noncytotoxic concentrations of milk chocolate extract slightly increased ROS formation after 1 hour of incubation, and this effect was more obvious in the case of 2 hours of incubation. Similarly, lower concentrations (0.1 and 0.5x) of semisweet and dark chocolate, as well as cocoa liquor extracts induced an increase in ROS formation, while the higher concentrations resulted with a significant decrease in ROS formation when compared to control. In cells treated with hydrogen peroxide, it can be observed that the final, highest concentrations of milk and semisweet chocolates, as well as cocoa liquor caused a decrease of ROS formation when compared to the control, as opossed to dark chocolate which increased the levels of ROS and indicated a prooxidant effect exerted by this extract. This might be due to the composition of dark chocolate and cocoa liquor, which contain the highest NFCS content, and consequently the highest polyphenol and methylxanthines content. The effect of this mixture of strong bioactives may be attributed for the observed prooxidant activity of dark chocolate and cocoa liquor extracts.

It has frequently been suggested that metal impurities can contribute to cell toxicity, by triggering ROS production by Fenton's reaction. Dietz et al. (1999) and Sahw et al. (2004) have reported that heavy metals, especially transition metals (Fe, Cu, Mn, etc.) induce oxidative stress in cells and tissues by transfering electrons directly in single-electron reactions, which generate free radicals. In our study, a high variability of toxic metal impurities in cocoa products was found using ICP-AES assays. As can be seen in table 1, dark chocolate and cocoa liquor contain higher contents of transition elements, which would imply on a potential for elevating and inducing ROS formation in HEp2 cells after the treatment with the cocoa extracts. Since dark chocolate and cocoa liquor which were characterized with the highest NFCS (and consequently the highest polyphenol, methylxanthines and mineral contents) also exhibited the highest growth inhibition (the lowest cell viability) and prooxidant activity of HEp2 cells, the results indicate a possible connection between the biological activity and bioactive content of cocoa products.

CONCLUSIONS

The results of this study revealed a strong relationship between the type of product/concentration/time of exposure and cytotoxic and antioxidant/prooxidant character of cocoa product extracts on HEp2 cells. Products containing the highest NFCS (cocoa liquor, dark chocolate) exhibited the most potent cytotoxic effect, as well as antioxidant properties (decrease of ROS formation). The highest concentrations of dark chocolate exhibited a prooxidant effect, which may imply on the effect of other bioactives present in cocoa products, especially methylxanthines and minerals whose effect on human cell lines subjected to oxidative conditions has not yet been clarified.

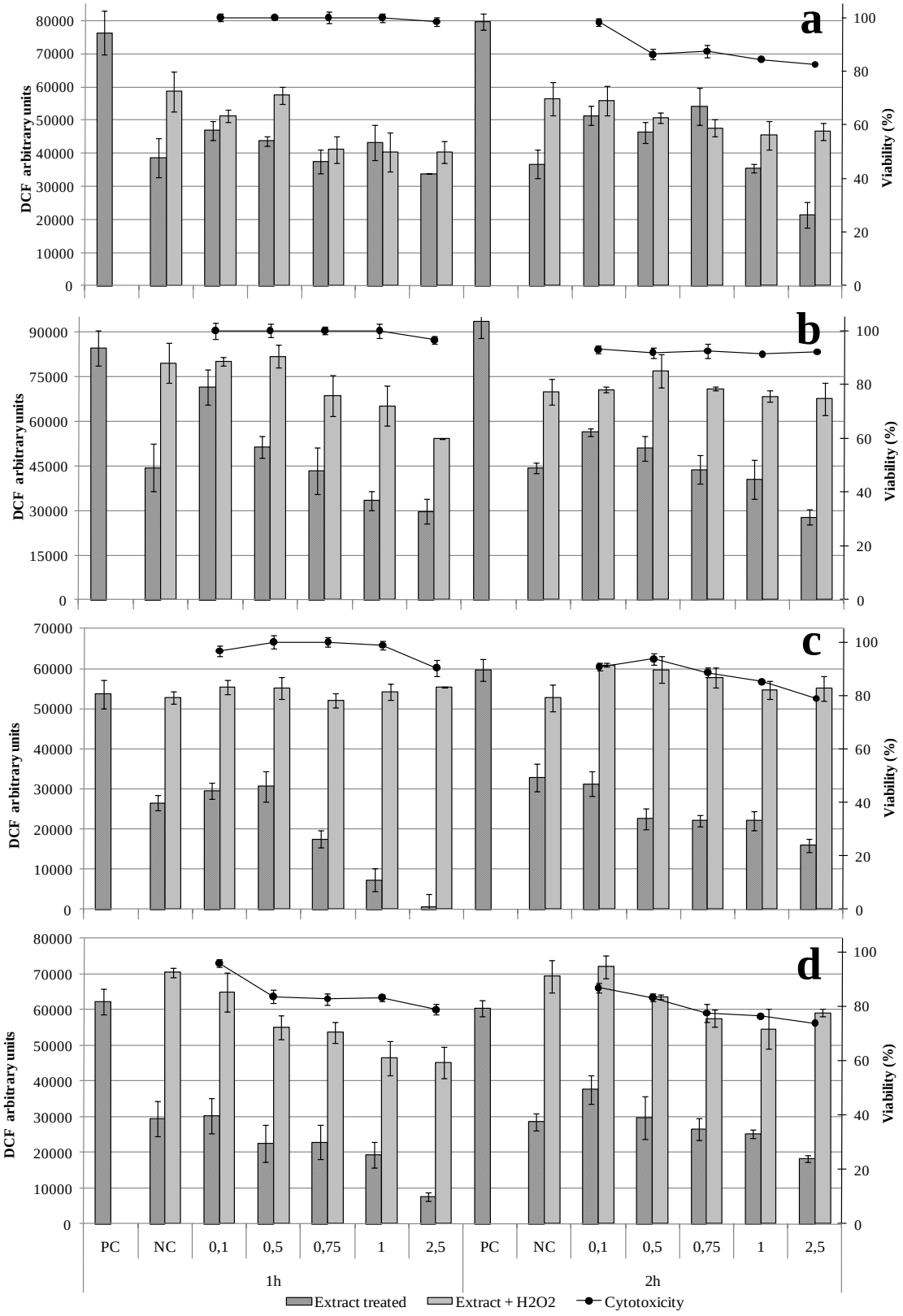

Figure 1. Survival and comparison of reactive oxygen species formation in HEp2 cells following 1h and 2h treatment with a) milk chocolate, b) semisweet chocolate, c) dark chocolate and d) cocoa liquor extracts with and without previous treatment with 250 μM hydrogen peroxide. Pooled data obtained from three experiments (the mean at the point +SD).

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science, Education and Sports, Republic of Croatia projects Nr. 058-0000000-3470 and 058-0582261-2246. The authors are grateful to Croatian food industry Zvečevo d.d. for supplying the cocoa product samples used in the study.

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MILK WITH FUNCTIONAL FOOD PROPERTIES

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ABSTRACT: This paper presents the results reflected on obtaining the milk enriched with omega-3 fatty acids. As source of omega-3 fatty acids, product of French company Vitalac called "TradiLin", was used. Trial was carried out on 20 trials (T) and 20 control (C) cows of Simmental breed and it lasted 80 days. Average content of omega-3 in trial and control group was 0.586 and 0.284 g/100g of extracted fat (P≤0.01), whereas content of omega-6 in same order of treatments was 3.141 and 3.011 g/100g of extracted fat (P≤0.05). Ratio omega-6/omega-3 fatty acids of 10.602 (control) was declined to 5.360 (trial), which is considered to be desirable from the aspect of nutrition with milk as functional food. Also, in other milk final product called "mladi kajmak", obtained by taking off milk fat from the surface of heat treated and cooled milk, content of omega-3 in trial and control group showed the same trend, 0.76 and 0.41 g/100g of extracted fat (P≤0.01), whereas content of omega-6 were 3.90 and 3.79 g/100g of extracted fat (P≤0.05). Ratio omega-6/omega-3 in control group was reduced of 9.24 to 5.10, which are considered to be extremely favourable. Chemical constituents of milk, somatic cell count as well as total plate count showed no differences between groups (P>0.05). Also, investigated treatment had no effect on changes in biochemical blood parameters.
Key words*: milk, omega-3 fatty acids, functional food*

INTRODUCTION

The term "functional food" includes those food stuffs which beside their main nutritive value contain such substances which have impact on improvement of general health condition, i.e. they have preventive and therapy effect (Sretenović, 2005).
In this trial as a source of omega-3 fatty acid, product known under the trade name "TradiLin", is used. The varieties of flax are specially selected for their richness in ALA omega- 3 fatty acids. "TradiLin" is produced from a fully patented extrusion technology which liberates the oils without denaturing them, so they can be digested more rapidly in such a way that it is available to the animal at the right time and at the right dose, inactivates the anti-nutritional factors (cyanogens) and preserves the fatty acid profile.
Typically 60% of fat intake for an average human comes from animal products (meat, milk, eggs, etc).These animal products have changed because the feeds that the animals now eat have changed. In the pass times 40 – 50 years ago, animals used to eat mainly forage (high omega -3, lower omega -6) but now typically eat less forage but consume more cereals and soya (high omega- 6, low omega -3) and as a result of that animal products (meat, milk, eggs etc) are much higher in omega -6 and lower in omega -3. So now the human diet, because of what animals are fed, is higher in omega -6 and lower in omega -3 i.e. traditional diets gave an omega -6: omega -3 ratio of 1:1, current human diets show a ratio of 20:1 and in animal diets up to 40:1.
Scientific studies suggest that this imbalance of omega- 6:omega -3 ratios is the cause of numerous physiological disorders and that better-balanced ratios could result in significant health improvements (Association Bleu-Blanc-Coeur, 2004).
In order to overcome numerous health problems, the food stuffs which aren't traditional sources of omega-3 fatty acids such as dairy products and pastry, meat, baby foods, etc. are enriching with moderate quantities of these fatty acids and there is increased demand for such products because of their well known positive effect on human health *(Sretenović et al., 2007a)*.

Since human organism has no enzymatic system necessary for synthesis of omega-3 fatty acids, they must be introduced through diet (they are called “essential fatty acids”).
Modern biotechnologies offer different ways for enriching of products with omega-3 *(Sretenović et al., 2007, Sretenović et al., 2009, Sretenović et al., 2009a)*. Products of animal origin such as milk, meat and eggs, enriched with omega-3 fatty acids are obtained by inclusion of sources of these acids into animal diets. Also, increase of the content of omega-3 fatty acids is achieved by application of modern biotechnological procedures in selection and plant breeding, by growing of varieties which synthesize higher quantity of ALA, i.e. fatty acids similar to EPA and DHA.
In connection with the above mentioned, goal of this research was to get milk and dairy products enriched with omega-3 fatty acids through the introduction of the “TradiLin” preparation in diets of lactating cows.

MATERIAL AND METHODS

Trial was carried out on 40 cows of Simmental breed, divided into two groups with 20 cows in each one. Both groups were made uniform according to relevant criteria, i.e. they were in the second lactation and with similar quantity of milk in previous lactation.
Source of omega-3 fatty acids was product “TradiLin” representing special type of flax processed using specific patented technological procedure.
In the laboratory of the Institute for Animal Husbandry the chemical composition of the product “TradiLin” was analyzed: 18.61% of total proteins, 9.78% of crude fibre, 5.68% of ash, 25.48% of crude fat with iodine number -172.29, g J/100g of fat; acid number -2.07, mg KOH/g of fat and peroxide number -2.66, mmol H_2O_2/kg of fat.
Trial lasted 80 days, i.e. administration of the preparation started in dry period 20 days before calving and lasted 2 months during lactation. Both groups of cows received identical diets and trial group received in the diet flax preparation in the quantity of 0.7 kg in dry period and 1,2 kg during lactation. Diets for cows in dry period consisted of: meadow hay 8 kg, silage of entire maize plant 15kg, concentrate for dry cows (14%UP) – 4.5kg. Fresh cows were fed diets of following composition: alfalfa hay 6 kg, silage of entire maize plant 10 kg, sunflower meal 1,5 kg and concentrate mixture (18%UP)-5.0 kg. Cows in lactation were fed diet consisting of alfalfa hay – 5,0 kg; silage of entire maize plant - 25 kg, maize ear silage – 5.5kg, sunflower meal – 3.5 kg kg and 2 kg of concentrate mixture (18%TP) for production of 30 kg of milk. Nutrition parameters of the diet for lactating cows were following: dry matter – 20.6 kg; 140.00 MJ (NEL); 3210 g total protein and 4302.2 g of crude fibre.
In the laboratory of the Institute for Animal Husbandry, Belgrade-Zemun, chemical composition of livestock feeds and preparation “TradiLin” was established, content of macro and micro elements, as well as content of organic acids in the silage. Following methods were used: total protein - SRPS ISO 5983:2001; moisture SRPS ISO 6496:2001; crude fat SRPS ISO 6492:2001; crude fibre SRPS ISO 6865:2004; ash SRPS ISO 5984:2002; calcium in livestock feeds AAS IS-LDM-14; calcium in premix - IS-LDM-7; phosphorus in premix IS-LDM-5; total phosphorus in feeds -IS-LDM-4; micro elements- AAS IS-LDM-14; sodium - Rulebook[A)] method 22; content of organic acids in the silage-IS-LDM-10; iodine number- SRPS ISO 3961:2001; peroxide number - SRPS ISO 3960:2001; acid number - SRPS ISO660:2000; level of acidity-volumetric method according by Soxhlet-Henkel.
Content of omega-6 and omega-3 fatty acids was done in the SP laboratory AD Bečej, using method MET 358 GC/MS SP.
Milk composition was measured by milkoscan apparatus. Somatic cell count was recorded according microscoping method (EN ISO 13366-1: 1997). The blood was withdrawn from jugular vein of lactating cows and biochemical parameters (glucose, protein, total bilirubin, aspartate aminotransferase-AST, alanine aminotransferase-ALT, total cholesterol, HDL and LDL cholesterol, calcium and phosphorus) were investigated from blood serum by automatic biochemical analyzer model Konelab 20.

The data from the experiment were developed statistically by using the computer program Statistica (Stat Soft Inc., Ver.6. 2003). Significance of mean differences was estimated by Student's t- test.

RESULTS AND DISCUSSION

Chemical composition of feeds and mineral content are presented in the Tab.1.

Tab. 1. Chemical composition of feeds

Parameter	**Feeds**						
	Alfalfa hay	Meadow hay	Maize silage	Maize ear silage	Concentrate (18%)	Sunflower meal	Premix
Total protein, %	15.01	6.61	2.07	5.25	18.88	36.44	-
Moisture, %	9.30	7.90	70.39	34.27	11.77	10.23	-
Crude fat, %	1.83	1.85	2.49	2.09	2.02	1.98	-
Crude fibre, %	32.96	33.02	6.77	4.90	7.60	15.45	-
Ash,%	7.11	10.65	1.47	1.00	6.36	6.78	-
Ca,%	1.20	0.29	1.5	0.28	1.02	2.92	-
P,%	0.29	0.42	0.6	1.43	0.73	9.95	-
NFE[6],%	33.79	39.97	16.81	52.49	53.37	29.12	-
Fe, mg/kg	-	-	-	-	-	-	1192
Cu mg/kg	-	-	-	-	-	-	1025
Mn, mg/kg	-	-	-	-	-	-	3555
Zn mg/kg	-	-	-	-	-	-	7348
Ca,%	-	-	-	-	-	-	20.32
P,%	-	-	-	-	-	-	3.84
Na,%	-	-	-		-		9.69

A) *Rulebook on methods of sampling and methods for physical, chemical and microbiological analysis of livestock feed (Official Journal SFRJ 15/87).*

Tab.2. Content of organic acids and reviewing maize silage according by Flieg

	Content	**Share%**	**Points**	**Assessment**
Butyric acid	0	0	50	VERY GOOD
Acetic acid	0.8351	19.45	18	
Lactic acid	3.4577	80.55	98	
Total:	4.2928	100.00	98	

From presented results it is obvious that all quality parameters varied within limits characteristic for specific feed.

In Tab 3. the contents of omega-6 and omega-3 in milk were presented and it is noticeable that the content of omega-6 fatty acids in trial group compared to the control one increased by 0.13g/100 g of fat, which was statistically significant at the level of (P≤0.05), whereas the content of omega-3 fatty acids in the same order increased by 0.302g/100 g fat which resulted in statistical significance at the level of P≤0.01. Ratio between omega-6 and omega-3 in milk in the trial group was 5.360, and was much more favorable than in the control one which amounted to 10.602 .

S similar situation regarding the content of fatty acids in the dairy product called "mladi kajmak". So, content of omega-6 fatty acids in trial group had increased by 0.11 g/100 g of fats (P≤0.05), whereas the content of omega-3 had increased by 0.350g/100g of fats, expressed through statistical significance it was P≤0.01.

Tab.3. Content of omega fatty acids and their ratio in milk and »mladi kajmak«

Milk	**Trial group**	**Control group**
Omega-6 fatty acids, g/100g of fat	3.141^{a}±0.1103	3.011^{b}±0.024
Omega-3 fatty acids, g/100g of fat	0.586^{A}±0.1632	0.284^{B}±0.1933
Ratio omega-6/omega-3 fatty acids	5.360	10.602
"Mladi kajmak"		
Omega-6 fatty acids, g/100g of fat	3.90^{a}±0.122	3.79^{b}±0.061
Omega-3 fatty acids, g/100g of fat	0.76^{A}±0.1123	0.41^{B}±0.421
Ratio omega-6/omega-3	5.10	9.24

All value expressed as mean ± Sd
a,b-values in rows with different letters differ significantly (P≤0.05)
A, B-values in rows with different letters differ significantly (P≤0.01

In Tab.4. durability of "mladi kajmak" is presented. Namely, "mladi kajmak" is declared as product with durability and taste which are maintained during 10 days in sale, and later the product undergoes fermentation and acquires characteristics of "stari kajmak". "Mladi kajmak" is characteristic and popular product on the territory of Serbia. In this product, in refrigerator's conditions the change in parameters of unsaturated fatty acids was monitored, i.e. inclination towards oxidation. Obtained results unambiguously indicate that "mladi kajmak" of the trial group enriched with omega-3 fatty acids, beside increased content of omega fatty acids has better durability compared to standard quality, since changes during the time occur slower, which was confirmed by lower values of peroxide number and level of acidity. In this way, nationally recognizable product which has been avoided by consumers due to health reasons and high content of saturated fats becomes foodstuff of high value with properties of functional food.

Tab.4. Durability of „mladi kajmak"

Parameter	**Sampling period, days**									
	Trial group					**Control group**				
	1	6	10	15	18	1	6	10	15	18
Iodine number,g J /100g fat	37.34					29.12				
Acid number, mg KOH/g fat	0.06					0.06				
Peroxide number,mmol H_2O_2/kg fat	1.08	1.14	1.44	1.46	1.58	1.12	1.23	1.54	1.56	4.88
Level of acidity, SH°	6.29	6.38	6.91	8.61	12.23	7.75	9.70	11.88	13.19	16.21

In Tab.5. chemical composition and quality of milk obtained from control and trial group are presented. Even though increased values of milk fat and proteins in trial group compared to control one were established, no statistical significance was exhibited because of high variations within groups. In both groups the somatic cell count, as well as total plate count, were at satisfactory level considering farm's conditions i.e. it is result of good managing system.

Tab.5. The chemical composition and bacteriological quality of milk

	Parameter/Parametar					
	Milk fat ,%	Protein,, %	Lactose,%	Dry matter without fat,%	Somatic cell count in 1ml	Total plate count in 1 ml
T	4.01^{a}±0.4359	3.72^{a}±0.2646	4.42^{a}±0.4899	8.82^{a}±0.7141	175660^{a}±233.48	<78000
C	3.80^{a}±0.3000	3.39^{a}±0.2449	4.74^{a}±0.2646	8.94^{a}±0.3464	208880^{a}±326.46	<78000

**Values in rows with same letters not differ significantly P>0.05*

In the beginning and in the end of trial, the blood was taken from v. jugularis and the most important blood parameters were recorded. At the beginning of trial there were no differences in biochemical blood parameters, and at the end of trial period, as it can be seen from Tab. 6, all of them were in physiologically optimal limits (regardless of the fact that in

trial group levels of bilirubin and ALT, which resulted in statistical significance) which indicated stable health condition of heads of cattle and also that the stress occurring in this most productive physiological stage has been overcome. Physiologically optimal values were compared according by Kaneko (1989).

Tab. 6. Biochemical parameters of the blood

Parameter	Groups		Reference range
	Trial	Control	
Glucose, mmol/l	2.74±0.3606	2.91±0.2646	2.5-4.2
Total bilirubin, mmol/l	4.46^{a}±2.2000	2.37^{b}±1.1916	0.2-8.5
Aspartate aminotransferase (AST), U/L	112.95 ±36.2202	124±51.7409	78-132
Alanine aminotransferase(ALT), U/L	23.15^{a}±9.2574	32.13^{b}±9.2574	14-38
Total protein, g/l	77.19±2.7731	78.02±8.4202	67.4-74.6
Total cholesterol, mmol/l	3.58±0.7616	3.73±0.6245	1.6-6.5
HDL cholesterol, mmol/l	2.27±0.4796	2.25±0.4123	
LDL cholesterol, mmol/l	1.20±0.3873	1.39±0.2449	
Calcium, mmol/l	2.21±0.1414	2.21±0.2000	2.4-3.1
Phosphorus, mol/l	2.12±0.4690	2.15±8.4202	1.8-2.1

a, b-values in rows with different letters differ significantly (P≤0.05)

Feeding dairy cows a ration containing whole flax seeds (linseed), flax (linseed) oil or milled, extruded or micronized linseed has five beneficial effects on the fat profile of cow's milk: (1) increasing the content of alpha-linolenic acid (ALA); (2) increasing the content of conjugated linoleic acid (CLA); (3) decreasing the omega-6/omega-3 ratio; (4) decreasing the overall saturated fat content; and (5) increasing the proportion of stearic acid relative to other saturated fatty acids. These enhancements to the fat profile of milk give consumers value-added foods with good sensory qualities and a healthier fat profile (Morris, 2009).

When results obtained in this study are compared with results obtained by other authors it can be said that they are in correspondence with them. *Oba et al. (2009).* obtained very important results by comparing the effects of α-linolenic acid in milk fat from Holstein cows which were fed diets supplemented by whole unprocessed, ground flax seed in the amount of 100g per kg of dry matter of diet. Problem occurs because of the specificity of digestion in ruminants where bio-dehydrogenization of unsaturated omega-3 fatty acids occurs, and in this way their digestibility is decreased unless they are made available, by processing procedure, to micro-organisms of rumen. Hypothesis that both feeding treatments influence the increase of concentration of α-linolenic acid in milk fat (8.3 and 8.6g/kg^{-1}) was confirmed and its concentration was three times higher in comparison to the one before the treatment, where instead of flax seed sunflower seed was used (2.6 g/kg^{-1}). Both treatments with whole unprocessed flax seed and ground seed indicate similar degree of absorption of α-linolenic acid in spite of lower digestibility of the whole unprocessed seed, which is noticeable by its content in faeces (259 vs. 129 g day^{-1}; $P < 0.001$). Ground flax seed doesn't increase significantly the absorption of α-linolenic acid in milk fat, since by increasing of its digestibility also the level of bio-dehydrogenization in rumen is increased.

Also, in research of *Petit (2002)* the effects of whole unprocessed flax seed as source of omega-3 fatty acids in diets for high yielding cows were studied and compared to other energy sources such as Megalac and micronized soy bean as energy source. It was concluded that inclusion of whole flax seed into diets for cows influenced not only the increase of quantity of milk (35.7kg/day compared to other two energy sources - 33.5 and 34.4kg/day), but also the protein content in milk as well as decrease of ratio of omega-6 and omega-3 fatty acids, which made it product of high quality from the aspect of human nutrition. Human diets rich in omega-6 fats result in a high omega-6/ omega-3 ratio. A high dietary omega-6/omega-3 ratio is linked with low-grade chronic inflammation that contributes to diseases such as Alzheimer disease, cancer, coronary heart disease, metabolic syndrome, obesity, type 2 diabetes, osteoporosis and even dry eye syndrome (Morris, 2007) Reducing

the omega-6/omega-3 ratio helps decrease inflammatory reactions and lowers the risk of chronic disease. A dietary ratio between 4:1 and 10:1 is recommended (Gebauer, 2006). Improving the ratio can be achieved by eating less omega-6 fats, eating more omega-3 fats or doing both. Buying milk and meat products with a low omega-6/omega-3 ratio helps improve the dietary mix of fatty acids in the human diet (Morris, 2009).

CONCLUSION

Including the whole flaxseed (patented procedure of special flax cultivar) into dairy cows diet has the beneficial effects on the fat profile of cow's milk. This is a good and simply way of obtaining milk with functional food properties. Having in mind that milk enriched with omega-3 fatty acids has a favorable effects on human and animal health, inclusion of flaxseed through product called "TradiLin" has a full justification.

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MINERAL CONTENT OF BUCKWHEAT ENRICHED WHOLEGRAIN WHEAT PASTA

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ABSTRACT: Light buckwheat flour (LBF) was used to substitute 20% of wholegrain wheat flour (WWF) in the formulation of wholegrain wheat pasta. Wholegrain wheat pasta (WWP) and buckwheat enriched wholegrain wheat pasta (BWWP) were produced on an industrial scale in the form of tagliatella.
Rheological test of control WWF dough and dough supplemented with LBF was used to predict the behaviour of dough using Mixolab. Results indicated that substitution of WWF with LBF in the formulation of pasta resulted in minor changes that did not influence the quality of enriched pasta.
Proximate composition, cooking quality and mineral content of BWWP were analysed and compared with those of WWP.
The substitution of WWF with LBF in the pasta formulation at the level of 20% resulted in significantly increased ($P < 0.05$) contents of P, Mg, K and Zn compared to WWP in dry pasta.
The reduction in mineral content of BWWP during cooking was significantly higher ($P < 0.05$) compared to WWP. The content of P, Mg and K were at same level in both type of pasta after cooking.
The obtained results suggest that enrichment of WWP with LBF at the level of 20% did not improve the mineral content of cooked pasta, although increase in minerals was observed in dry pasta.
Key words: *pasta, light buckwheat flour, dough rheology, Mixolab, mineral content*

INTRODUCTION

Pasta products are largely consumed all over the world and they are frequently manufactured from wheat flour, which is known to be the best raw material suitable for its production. The best properties of wheat flour for pasta production result from protein structure of wheat that enables to form a matrix with encapsulated starch granules. On the other hand, wheat flour is characterized with relatively low lysine, methionine and treonine content, as well as some minerals and vitamins (Heger and Frydrych, 1987). Fortification of wheat pasta in order to improve its nutritional quality and to produce functional pasta product is described in literature (Bahnassey et al., 1986; Rayas-Duarte et al., 1996; Shogren et al., 2006).
Buckwheat (Fagopyrum esculentum Moench) is an important pseudocereal known as a dietary source of protein containing high levels of essential amino acids (Bonafaccia et al., 2003b), starch and dietary fibres (Skrabanja et al., 2004), essential minerals (Steadman et al., 2001) and trace elements (Bonafaccia et al., 2003a). This crop also contains antioxidant compounds (Dietrych-Szostak and Oleszek 1999), first of all rutin (Holasava et al., 2002), being responsible for benefitial effect on health. Regular consumption of buckwheat containing products reduces the risk of high blood pressure, prevents oedema and hemorrhagic diseases, prevents diabetes and reduces the risk of arteriosclerosis (Ihme et al., 1996; Kim et al., 2004).
Buckwheat flour has been used for supplementation of wheat pasta in order to produce functional pasta with nutritional benefits (Bilgiçli, 2009a; 2009b; Bonafaccia and Kreft, 1994; Chillo et al., 2008; Hung et al., 2007). Furthermore, buckwheat bran has been incorporated into spaghetti formulation to improve its quality (Manthey et al., 2004; Manthey and Hall, 2007).

The majority of research of buckwheat enriched pasta was focused on its mechanical strength and cooking quality related to decreased gluten matrix (Hung et al., 2007; Rayas-Duarte et al., 1996), and sensory properties (Chillo et al., 2008; Rayas-Duarte et al., 1996).
The production and cooking of pasta influence its nutritional quality. Therefore, the supplementation of pasta with functional ingredient(s) has to be followed by availability of added compounds that contribute to desirable nutritional benefits. The loss of amino acids, i.e. lysine, during processing (Dexter et al., 1984), as well as some minerals (Manthey and Hall, 2007) and polyphenols (Verardo et al., 2011) during cooking of pasta has been reported.
Knowing that production and cooking influence the mineral content of pasta, the aim of this work was to investigate if there is a possibility to produce buckwheat enriched wholegrain wheat pasta with acceptable properties from the rheological point of view, and to determine the effect of processing and cooking on mineral content of pasta. The obtained information would be useful for optimization of process parameters that would provide the stability of nutritional components at the time of pasta consumption.

MATERIAL AND METHODS

Raw materials

Light buckwheat flour (LBF) was obtained from Hemija Commerce, Novi Sad, Serbia, and wholegrain wheat flour (WWF) was purchased from Žitko, Bačka Topola, Srbija.

Pasta dough formulation

Wholegrain wheat pasta (WWP) was produced using WWF and buckwheat enriched wholegrain wheat pasta (BWWP) was obtained by substitution of WWF with LBF at the level of 20%. WWF or WWF-LBF mixture was hydrated with deionised water to 320 g kg^{-1} absorption in order to achieve proper dough consistency (Manthey et al., 2004).

Rheological characteristics of pasta dough

The rheological behaviour of both prepared dough formulations was examined using Mixolab (Chopin, Tripette et Renaud, Paris, France). All measurements were performed using the modified Mixolab “Chopin +” protocol (ICC, 2006). Modification of “Chopin +” protocol is due to dough mass increase from 75 g to 90 g because of the specific nature of the buckwheat flour. Instrumental settings were: initial mixing at 30 °C for 8 min, heating to 90 °C during 15 min at a heating rate 4 °C/min, holding at 90 °C for 7 min, cooling to 50 °C at a 4 °C/min cooling rate and holding for 5 min at 50 °C. Mixing speed was kept constant during the measurement at 80 rpm.

Industrial pasta production

Two types of pasta (WWP and BWWP) were produced on an industrial scale by using Ital past Mac 60 (Parma, Italy). WWF or WWF-LBF mixture was hydrated and mixed in pre-mixer for 12 min. After that, the entire quantity was transferred to a mixer and mixed for 6 minutes. The obtained dough was extruded at the extrusion speed of 42 rpm as tagliatella for 42 min. The extruded tagliatella was dried in a dryer (Ital past D200) for 13.5 h at the temperature of 41.3 °C.

Proximate composition

Proximate composition of WWF, LBF, WWP and BWWP was analyzed using AOAC methods (1984) for determination of moisture, crude protein, ash, crude cellulose, crude fat and starch content.

Mineral composition

Mineral composition (P, Mg, K, Zn, Fe and Mn) of WWF, LBF, WWP and BWWP (uncooked and cooked) was determined using a Varian Spectra AA 10 (Varian Techtron Pty Limited, Mulgvare Victoria, Australia, 1989) atomic absorption spectrophotometer equipped with a

background correction (D2-lamp). The sample preparation consisted of a dry ashing procedure at 450 °C as described by Pavlović et al. (2001).

Pasta cooking quality

Optimal cooking time, cooking loss and volume increase of WWP and BWWP were determined according to AACC methods (1995).

Statistical analysis

All analyses were performed in triplicate, and the mean values with the standard deviations (S.D.) are reported. Analysis of variance and Duncan's multiple range test were used. Statistical data analysis software system STATISTICA (StatSoft, Inc. 2008). *P* values < 0.05 were regarded as significant.

DISCUSSION AND RESULTS

Proximate composition of flours

Proximate composition of the commercially available WWF and LBF are presented in Table 1.

The protein content was significantly higher (*P* < 0.05) for LBF compared with WWF, followed by higher ash and fat content. The results obtained in the present study are comparable with the published data (Alvarez-Jubete et al., 2009; Bonafaccia et al., 2003b; Steadman et al., 2001).

Also, content of P, Mg, and K in LBF was found to be much higher compared to WWF (Table 1). The obtained mineral contents of WWF and LBF are within the ranges reported by Ikeda et al. (1995), Steadman at al. (2001) and Bonafaccia et al. (2003a).

Table 1. Proximate composition (%, dry basis) and minerals (mg/100 g) of wholegrain wheat flour (WWF) and light buckwheat flour (LBF)

Proximate composition	WWF	LBF
Moisture	11.7 ± 0.02[a]	12.1 ± 0.02[b]
Crude protein (Nxfactor)[a]	13.6 ± 0.12[a]	15.8 ± 0.19[b]
Ash	1.46 ± 0.01[a]	2.00 ± 0.0[b]
Crude cellulose	1.55 ± 0.03[b]	0.87 ± 0.01[a]
Crude fat	1.52 ± 0.02[a]	2.49 ± 0.11[b]
Starch	66.6 ± 0.18[b]	58.5 ± 0.01[a]
Minerals		
P	253 ± 4.26[a]	447 ± 18.15[b]
Mg	81.9 ± 1.38[a]	177 ± 1.77[b]
K	272 ± 5.38[a]	535 ± 4.68[b]
Zn	2.09 ± 0.11[a]	2.38 ± 0.02[b]
Fe	3.51 ± 0.09[a]	4.20 ± 0.08[b]
Mn	2.79 ± 0.11[b]	1.72 ± 0.02[a]

[a] *– Nitrogen-to-protein conversion factors are: 5.7 for WWF and 6.25 for LBF.*

Values are means of three determinations ± standard deviation.

Values of the same row with the same superscript are not statistically different (P < 0.05).

Rheological characteristics of pasta dough

Physical and chemical changes take place at micro structural levels in dough when some part of wheat flour in the formulation is replaced with another type of flour. It has already been concluded that rheological tests on dough can predict its behaviour during production on an industrial scale (Torbica et al., 2010). Therefore, the rheological properties of wholegrain wheat dough and dough enriched with LBF were investigated using the Mixolab (Table 2).

Table 2. Mixolab properties of dough systems made by using wholegrain wheat flour (WWF) and mixture of 80% wholegrain wheat flour and 20% light buckwheat flour (80WWF-20LBF)

Dough type	Development time (min)	Stability time (min)	C2 torque (Nm)	C3 torque (Nm)	C3-C4 torque (Nm)	C5-C4 torque (Nm)	Water absorption (%)
100WWF	8.39[a]	7.91[a]	0.51[a]	2.20[a]	0.51[b]	0.78[a]	62.6[a]
80WWF-20LBF	8.50[a]	8.05[a]	0.53[a]	2.15[a]	0.29[a]	0.77[a]	63.6[a]

Values are means of three determinations ± standard deviation.
Values of the same column with the same superscript are not statistically different (P < 0.05).

Based on the results presented in Table 2 it can be concluded that rheological behavior of two investigated systems is quite similar. This enabled the production of buckwheat enriched wholegrain wheat pasta (BWWP) in industrial conditions without modifying equipment and process parameters of traditional pasta production.

Proximate composition of pasta

Proximate composition of WWP and BWWP are presented in Table 3. The pasta containing LBF is superior in protein, ash and fat content than WWP due to higher content of these nutrients in LBF compared to WWF (Table 1).

Table 3. Proximate composition (% dry basis) of wholegrain wheat pasta (WWP) and buckwheat enriched wholegrain wheat pasta (BWWP)

Pasta sample	Moisture	Crude protein	Ash	Crude cellulose	Crude fat	Starch
WWP	11.3 ± 0.03[b]	13.1 ± 0.11[a]	1.30 ± 0.03[a]	1.46 ± 0.01[b]	1.30 ± 0.01[a]	65.9 ± 0.18[b]
BWWP	11.1 ± 0.01[a]	14.1 ± 0.08[b]	1.59 ±0.02[b]	1.36 ± 0.02[a]	1.58 ± 0.08[b]	64.1 ± 0.01[a]

Values are means of three determinations ± standard deviation.
Values of the same column with the same superscript are not statistically different (P < 0.05).

Pasta cooking quality

Optimal cooking time was shorter for BWWP when compared to WWP (Table 4). This may be due to the physical disruption of gluten matrix which provided water absorption into BWWP. Similar observations were reported by Manthey et al. (2004) and Chillo et al. (2008). The weakening of gluten matrix in BWWP caused the significant increase in cooking loss of enriched pasta (Table 4). Alamprese et al. (2007) and Bilgiçli (2009b) also found that matter loss in cooking water was higher in pasta containing buckwheat.

Moreover, Alamprese et al. (2007) detected that buckwheat containing pasta had a significantly higher weight increase during cooking than wheat pasta. The authors explained this observation underlining the high non-starch polysaccharide content of buckwheat with high water absorption capacity and the structure of buckwheat starch granules with irregular structure containing more amorphous areas than those of wheat. The disruption of gluten matrix in buckwheat containing pasta could also promote greater water absorption in comparison with wheat pasta (Manthey et al. 2004). However, the volume increase for BWWP is not significant probably due to the low substitution level in this pasta formulation.

Table 4. Cooking quality of wholegrain wheat pasta (WWP) and buckwheat enriched wholegrain wheat pasta (BWWP)

Pasta sample	Optimal cooking time (min)	Cooking loss (%)	Volume increase
WWP	9.10 ± 0.32[b]	7.83 ± 0.65[a]	2.86 ± 0.24[a]
BWWP	8.05 ± 0.12[a]	9.33 ± 0.40[b]	3.07 ± 0.46[a]

Values are means of three determinations ± standard deviation.
Values of the same column with the same superscript are not statistically different (P < 0.05).

Mineral content of pasta

Mineral contents of WWP and BWWP are presented in Table 5. The substitution of WWF with LBF in the pasta formulation at the level of 20% resulted in significantly increased ($P < 0.05$) contents of P, Mg, K and Zn, which were expected considering the mineral content of WWF and LBF (Table 1). The high K, Mg and P contents of buckwheat flour increased the mineral content of tarhana (Bilgiçli, 2009a), gluten-free tarhana (Bilgiçli, 2009c) and eriste (Bilgiçli, 2009b) which were produced following the modified formulations that included buckwheat flour instead of a part of wheat flour.

Table 5. Effect of cooking on mineral content (mg/100 g dry basis) of wholegrain wheat pasta (WWP) and buckwheat enriched wholegrain wheat pasta (BWWP)

Pasta sample	P	Mg	K	Zn	Fe	Mn
Uncooked						
WWP	268 ± 0.83[a]	94.5 ± 2.81[a]	277 ± 0.32[c]	2.03 ± 0.01[a]	3.92 ± 0.12[a]	3.53 ± 0.14[a]
BWWP	345 ± 16.5[b]	125 ± 5.20[b]	402 ± 8.48[d]	2.32 ± 0.01[b]	4.09 ± 0.28[a]	2.82 ± 0.07[b]
Cooked						
WWP	253 ± 12.1[a]	96.8 ± 2.62[a]	47.7 ± 0.02[a]	2.06 ± 0.10[a]	3.92 ± 0.10[a]	3.58 ± 0.08[a]
BWWP	268 ± 7.39[a]	96.0 ± 1.09[a]	68.6 ± 1.87[b]	2.43 ± 0.16[b]	4.08 ± 0.07[a]	2.87 ± 0.09[b]

Values are means of three determinations ± standard deviation.
Values of the same column with the same superscript are not statistically different ($P < 0.05$).

Processing of dough into both types of pasta (WWP and BWWP) had little or no effect on mineral content (Table 1 and 5). This finding was in accordance with observation of Yaseen (1993) and Manthey and Hall (2007), who reported that pasta processing did not influence the mineral composition of pasta.

During cooking some amount of material is released into the cooking water. The loss of materials for nontraditional pasta is greater than for wheat pasta due to weak gluten matrix of nontraditional pasta that permits a greater leaching of nutrients into the cooking water (Duarte et al., 1996; Resmini and Pagani, 1983). The reduction in mineral content of BWWP during cooking was significantly higher ($P < 0.05$) compared to WWP (Table 5). The reduction in P, Mg and K in BWWP was of 22%, 23%, and 88%, respectively. Contrary, there were no significant reduction in P and Mg for WWP, but the reduction in K was 83%. These results are in agreement with average reduction in minerals of about 28% in spaghetti containing 25% buckwheat bran flour published by Manthey and Hall (2007). These authors reported that the major reduction was detected for K (62%). Other literature data suggest that the decline of 10-30% in minerals during cooking of traditional pasta was typical, with the exception of K that leached out by 67% (Ranhotra et al., 1985; Yaseen et al., 1993).

In contrast, the contents of Zn and Fe in both types of pasta were unaffected by cooking (Table 5). In general, the increased contents of P, Mg and K in enriched dry pasta that had been achieved by substitution of WWF with LBF were decreased and reached the same levels as in WWP after cooking. That implies that enrichment of WWP with LBF at the level of 20% did not improve the mineral content of cooked pasta, although increase in minerals was observed in dry pasta.

CONCLUSIONS

The substitution of WWF with LBF in the pasta formulation at the level of 20% resulted in significantly increased ($P < 0.05$) contents of P, Mg, K and Zn compared to the control pasta (WWP). The increased contents of these minerals in buckwheat enriched wholegrain wheat pasta (BWWP) declined and reached the same levels as in WWP after cooking.

The obtained results suggest that enrichment of WWP with LBF did not result in significant improvement in mineral content of cooked pasta, although increase in minerals was observed in dry pasta.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31029 financed by the Ministry of Education and Science, Republic of Serbia.

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ENHANCEMENT OF POMEGRANATE JUICE QUALITY THROUGH THE COMBINATION OF THERMAL TREATMENTS AND BLENDING WITH LEMON JUICE

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ABSTRACT: Consumer acceptance of healthy food is subordinated to quality and sensory properties. Nevertheless, red fruit juices can display unpleasant and unacceptable brown colour because of thermal treatments. As an attempt to solve these browning problems in pomegranate juices, the effect of two different heat treatments on the phytochemical composition and colour parameters of pure and blended juices was assessed through storage. *Mollar de Elche* varietal juice was mixed with lemon juice in order to assess the effect of blending on *Mollar de Elche* preservation. Juices were heat treated at 65ºC for 30 s (LTLT pasteurization) or 90ºC for 5 s (HTST pasteurization). Colour parameters and bioactive phytochemicals (individual anthocyanins and vitamin C) were monitored. Blended juices showed a protective effect on colour even though high falls in anthocyanins were recorded. Vitamin C was kept in those juices containing lemon but not in pure pomegranate juices. Heat processing had a remarkable effect on the blend of pomegranate with lemon as LTLT-treatment preserved the bioactive composition much better than HTST. Overall, blended juices protected juice characteristics in a greater extent than *Mollar de Elche* juice.
Key words: *Pasteurization, anthocyanins, ascorbic acid, colour,* Punica granatum *L.*

INTRODUCTION

Pomegranate (*Punica granatum* L.) has been asserted as a natural source rich in bioactive phenolics, such as anthocyanins and ellagitannins, with a myriad of health promotion features (Mena et al., 2011a). Pomegranate fruits are usually earmarked for direct fresh consumption and are also processed by food industry for the elaboration of juices, wines, and jams, among other products. Nevertheless, pomegranate industrialization may display complications since processing stages alter the phytochemical composition and quality parameters of juices (Ferrari et al., 2010; Mikkelsen and Poll, 2002; Yildiz et al., 2009). Likewise, although pasteurization treatments are usually used to preserve pomegranate juices and extend their shelf-life, heat processing varies the aroma profile and result in colour degradation (Maskan, 2006). Consequently, this decline of important marketable quality attributes leads to consumer's disapproval and falls in product sales. Actually, browning and colour alteration are the main problems of some slightly coloured pomegranate varieties as *Mollar de Elche*, cultivar widely grown in Spain (Mena et al., 2011b). Therefore, solving colours alterations of pomegranate juices during storage is the major sticking point for pomegranate juice processing.

Different technological approaches are being developed and could be regarded as promising ways of pomegranate quality preservation (Ferrari et al., 2010; Yildiz et al., 2009); nonetheless, thermal pasteurization is still regarded as the most rentable preservation process for being inexpensive, efficient, and environmentally friendly (Silva and Gibbs, 2010). Then, attending to the widespread use of thermal pasteurization and the industrialization problems for some pomegranate varieties aforementioned, novel and combined solutions should be performed. In this sense, blends of pomegranate with other fruit juices have been emphasized as successful ways to improve and preserve the phytochemical quality of pomegranate juices through storage (González-Molina et al., 2009;

Waskar, 2011). However, the effects of heat treatments in these promising blended juices have not been assayed yet despite they could remarkably alter juice quality.
The aim of the present work was to solve browning problems in slightly coloured pomegranate juices at thermal processing. For that, the effect of two different heat treatments (high temperature-short time and low temperature-long time) on the phytochemical composition and colour parameters of different juices made from cultivar *Mollar de Elche* were assessed for along juices shelf-life. In this way, a pure *Mollar de Elche* varietal juice and a blend of *Mollar de Elche* (75%) plus lemon juice (25%) were compared, after pasteurization, during storage. Likewise, these assays provided basic information on how thermal treatments affect browning developing according to juice composition and storage conditions.

MATERIAL AND METHODS

Mollar de Elche varietal juice was mixed with lemon juice (75%M+25%L, labelled as ML). Likewise, *Mollar de Elche* 100% (labelled as M100) was kept also as control juice. Juices were heat treated at 65 ºC for 30 s (LTLT, low temperature-long time pasteurization) or 90 ºC for 5 s (HTST, high temperature-short time pasteurization) in a semi-tubular pasteurizer 25 L/h (Mipaser Prototype). Later, juices were aseptically placed in screw capped plastic vials (15 mL) and stored in darkness at 25 ºC for 42 days. Triplicate solutions were prepared for each juice.

Colour measurement

Colour measurement was determined after centrifugation as in Mena et al. (2012). Data (CIEL*, *Chroma*, and *Hue* angle) were recorded and processed using the Minolta Software Chromacontrol S, PC-based colorimetric data system.

Identification and quantification of main bioactive phytochemicals

Chromatographic analyses were carried out on Merck-Hitachi liquid chromatograph equipped with a DAD UV–vis L-7455 (Tokyo, Japan). Chromatograms were recorded and processed on a Merck-Hitachi D-7000 HSM PC based chromatography data system. A 20 µL sample was analysed on a Luna C18 column (25 cm x 0.46 cm, 5 µm particle size; Phenomenex, Macclesfield, UK). Chromatographic conditions are described in Pérez-Vicente et al. (2004). UV chromatograms were recorded at 520 nm and chromatographic comparison was used to identify anthocyanins previously reported (González-Molina et al., 2009). Anthocyanins were quantified as cyanidin 3-glucoside by the absorbance of their corresponding peaks.
Ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents were determined by HPLC-UV as described elsewhere and fully detailed in González-Molina et al. (2008). The vitamin C content was calculated as the sum of AA and DHAA.

RESULTS AND DISCUSSION

Characteristic colour parameters of M100 were improved by the addition of lemon juice to *Mollar de Elche* varietal juice (blend ML) (Table 1) (González-Molina et al., 2009). Regarding the influence of thermal treatments on colour parameters during storage (Figure 1), slight increases were displayed in lightness (CIE*L*) for all juices as it has been already reported for untreated juices (González-Molina et al., 2009; Pérez-Vicente et al., 2004). Likewise, M100, both LTLT- and HTST-treated, changed significantly its colour parameters during storage (Figure 1), rendering juices highly browned (higher *Hue* angle) and with a lesser colour intensity (lower *Chroma*). Similar colour alterations were also recorded for unpasteurized pomegranate juice by Martí et al. (2002). On the contrary, blended juices displayed superior colour stability rather than pure *Mollar de Elche* juice. Actually, although variations in *Chroma* and *Hue* angle of ML juices were registered, these changes resulted in minor losses of colour purity and hue when compared to M100. Therefore, blended juices were found to

possess an appealing colour along storage with reduced browning and chromatic characteristics close to the effective juice colour of pomegranate juices (Yildiz et al., 2009). With regard to the treatment effect along storage, pure pomegranate juices (M100) did not reveal significant variations between both heat processing (LTLT and HTST). However, ML-LTLT preserved the colour properties of ML juices much better than ML-HTST by reducing *Hue* increases through storage and, hence, pointing out the importance of heat treatment. In fact, ML-LTLT tendency was quite similar to that recorded for unpasteurized lemon-pomegranate juices where browning occurrence was attenuated because of mixes (González-Molina et al., 2009). Polymerization, anthocyanins degradation, and non-enzymatic browning may be the responsible reactions behind the colour deterioration of red juices (Maskan, 2006).

Table 1. Colour parameters and bioactive phytochemicals of the raw juices

Raw juice	Colour parameters			Bioactive phytochemicals (mg/100mL)	
	CIE*L*	*Chroma*	*Hue* angle	Antocyanins	Vitamin C
M100	75,49 ± 0,14 [a]	28,37 ± 0,72	-4,11 ± 0,08	16,61 ± 0,41	8,86 ± 0,73
ML	68,28 ± 0,22	46,50 ± 0,42	-1,19 ± 0,03	13,37 ± 0,42	8,50 ± 0,75
p-value	* * * [b]	* * *	* * *	* * *	n.s.

Statistical treatment notes: [a] Values are expressed as means of triplicate determinations ± standard deviation [b] Significant differences ($p < 0.001$) between raw juices parameters were determined by Student's t-test.

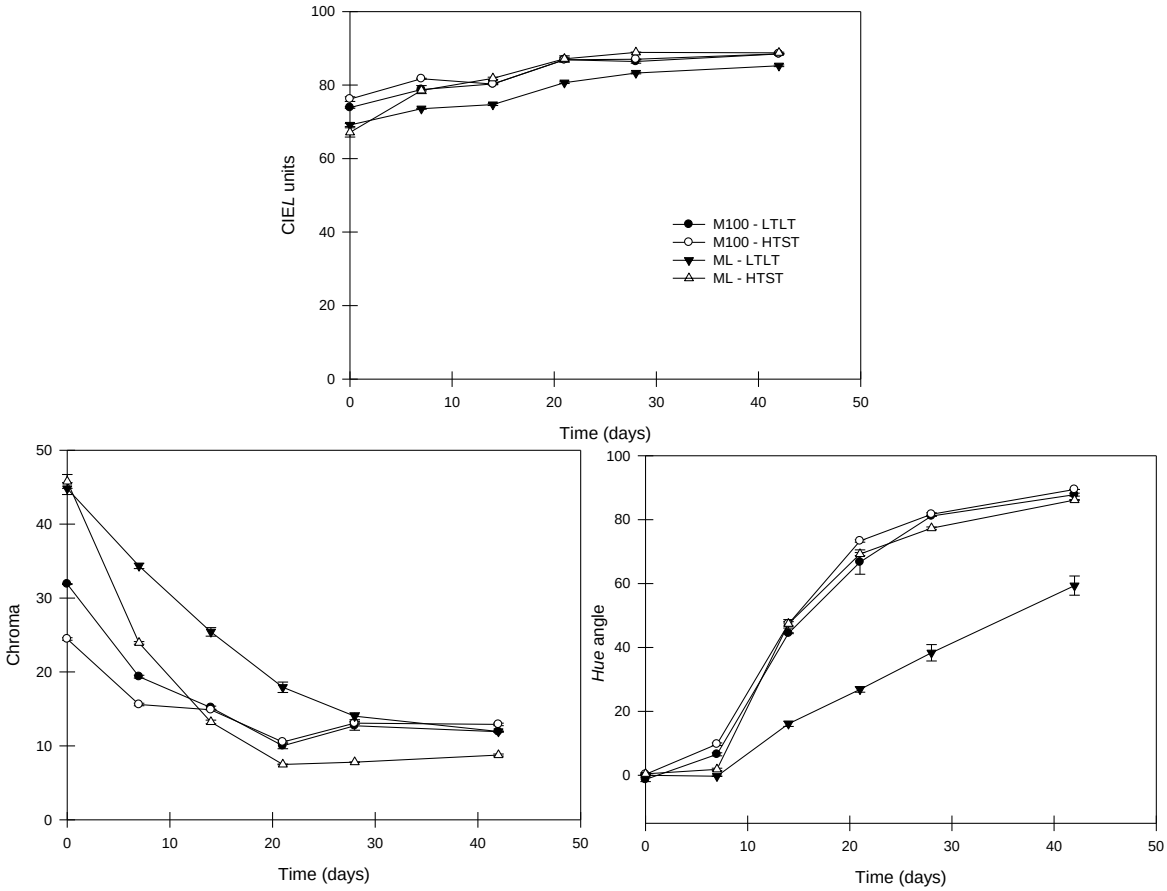

Figure 1. Evolution of colour parameters through storage of pasteurized pomegranate juices

With regard to anthocyanins, the phenolic pigments responsible for the red colour of pomegranate juices, decreases in their concentration during storage are displayed in Figure 2. Drops in anthocyanins were marked by the kind of pasteurization used. Likewise, Mikkelsen and Poll (2002) emphasized how the extent of thermal treatment may affect anthocyanins stability owing to enzymes inactivation. In fact, HTST-treatment resulted in two-folds more pigment destruction than LTST-treatment in ML juice, justifying the severe colour alterations of ML-HTST aforementioned. Decreases in anthocyanins of pure pomegranate juices (M100-LTLT and M100-HTST) did not undergo important differences with respect to the thermal treatment used. Likewise, anthocyanins decreases were well correlated to *Chroma* variations ($r = 0.79$, $p < 0.001$) but not to *Hue* angle (no strong correlation was established) (Alighourchi and Barzegar, 2009; Fischer et al., 2011). Consequently, reductions in anthocyanin content would be responsible for the *Chroma* variations showcased herein for all the juices.

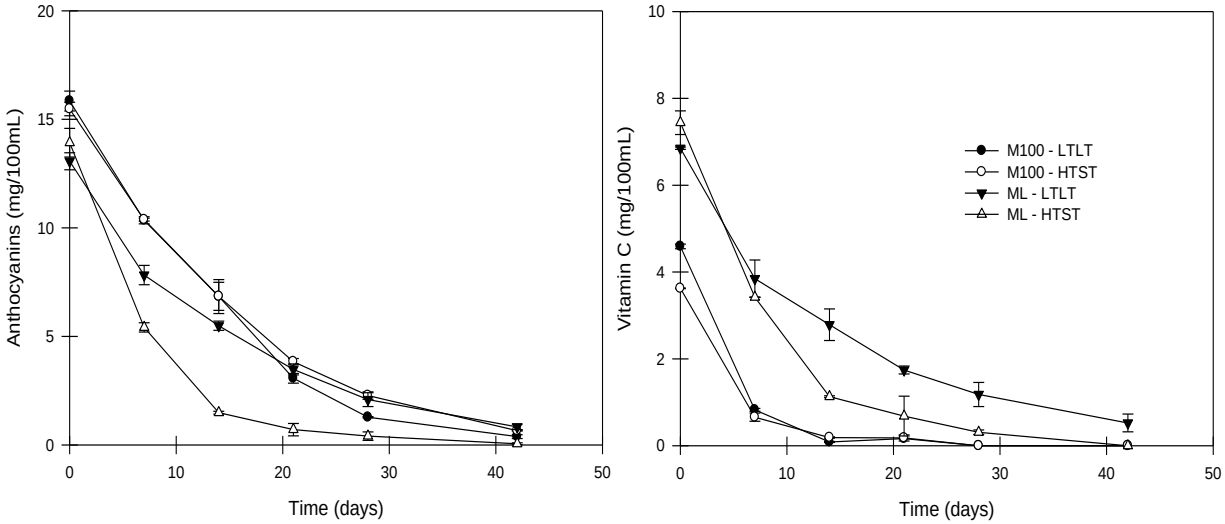

Figure 2. Evolution of anthocyanins and vitamin C through storage of pasteurized pomegranate juices

Concerning vitamin C evolution through storage, the full degradation of this vitamin was registered for M100 at the beginning of the storage (Figure 2). ML juices presented always the lowest degradation rates and, thus, vitamin C in pomegranate-lemon blends (ML-LTLT and ML-HTST) was more stable than in pure pomegranate juices (M100), as expected (Martí et al., 2002). González-Molina et al. (2009) pointed out that vitamin C of lemon juice was preserved when mixed with pomegranate juice and a similar protective effect of vitamin C by combining juices was here noticed. Regarding the thermal treatment influence, LTLT protected the vitamin C content of juices in a greater extent than HTST. Likewise, it should be mentioned that ML-HTST juice had remarkably variations in anthocyanins and colour parameters besides the strong vitamin C decreases. Consequently, the protective role of anthocyanins in vitamin C preservation (González-Molina et al., 2009) was not observed for this treatment although the concomitant degradation of vitamin C and anthocyanins could account for this result (García-Viguera and Bridle, 1999). Actually, the mutual degradation of both phytochemicals may explain how higher vitamin C degradation rates are correlated well with higher anthocyanins degradation rates ($r = 0.81$, $p < 0.01$). Moreover, the degradation of both phytochemicals may boost browning process (Maskan, 2006) as colour parameters reflected for ML-HTST. On the contrary, lower losses of both vitamin C and anthocyanins in ML-LTLT juice as well as minor colour variations would assert the role of phytochemicals degradation in browning.

CONCLUSIONS

Pasteurization of pomegranate juice mixed with lemon showed to protect the juice characteristics of pasteurized *Mollar de Elche* juices. In fact, blended juices preserved the chromatic composition of slightly coloured *Mollar de Elche* juices as well as enhanced and/or retained their bioactives. Moreover, a specific pasteurization treatment should be applied for each juice in order to achieve the highest quality of rich-in-phytochemicals pomegranate juices.

ACKNOWLEDGEMENTS

The authors wish to thank Fundación Agroalimed for the financial support of this work (Project “Producción de Zumo de Caqui e Industrialización de Granada”). PM was funded by a grant of the FPU Fellowship Programme from the Spanish Ministry of Education. This work was also partially supported the Ibero-American Programme for Science, Technology and Development (CYTED) - Action 112RT0460 CORNUCOPIA.

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THE INFLUENCE OF APPLE JUICE ADDED IN BLACKBERRY AND SOUR CHERRYJUICES ON THEIR TOTAL PHENOLS CONTENT AND ANTIOXIDATIVE CAPACITY

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ABSTRACT: The study is based on the investigation of total phenol contents and antioxidant potential of blackberry and sour cherry juices blended with apple juice. The objective was to determine the effect of apple juice added in different amount on total polyphenols and antioxidant activity of blended mixtures. Blackberry, sour cherry and apple juices were made from concentrated fruit juices. Apple juice was used for the correction of acidity. The content of the total polyphenols in fruit juices was determined using the Folin-Ciocalteu reagent while the antioxidant activity was investigated using FRAP method. The total polyphenols content as well as antioxidative activity were gradually decreased in all tested mixture with the increasing amount of apple juice. However, it was concluded that the mixture of blackberry or sour cherry juice with apple juice was sensory more acceptable than the juices obtained from only one type of small fruit.
Key words: *blackberry, sour cherry, apple, juice, total polyphenol, antioxidative capacity*

INTRODUCTION

Berries such as blackberry and sour cherry are the fruits which can be eaten either as a fresh fruit or as processed food in juices, jams, dried fruit, ice cream, etc (Amakura et al., 2000). These fruits are among the most important sources of polyphenol compounds in our diets, especially hydroxybenzoic and hydroxycinnamic acid derivatives, anthocyanins, flavonols, catechins, and tannins (Manach et al., 2004), which continue to be present in their processed products (Heinonen et al., 1998). The significance of the antioxidant properties of foods in the maintenance of health and in protection from degenerative diseases is of growing interest among scientists, food manufacturers, consumers, and health organizations (Müller et al., 2010; Obón et al., 2011; Bermudez-Soto &Tomas-Barberan, 2004). The Food Standards Agency (FSA) has established that increasing consumption of fruits and vegetables to 5 or more daily servings provides a desirable intake of antioxidants and improves human health (FSA, 2010). For this reason, there is a trend in the food industry toward functional foods, with health promoting effects based on their antioxidant properties (Mazza et al., 2002; Wootton-Beard & Ryan, 2011). In addition, the results of *in vivo* experiments have shown that the consumption of small fruit juices contributes to maintaining of human health (Netzel et al., 2002;García-Alonso et al., 2006).
Fruit juice concentrate is the result of an industrial process of evaporation of excess water by heat treatment in order to obtain the product with better conditions for storage, transport, and preservation. According to some authors processing of food can increase flavonol levels in the food (Stewart et al., 2000). Fruit juice processing can increase the flavonoid content because extraction processes can release flavonoids from the peel, and these flavonoids might be better absorbed than those from the fresh fruits. Therefore, small fruit juice concentrates could be very suitable for the production of functional foods.
In this study blackberry and sour cherry juices were made from the concentrate fruit juices. However, juices of small fruits cannot be consumed in large quantities due to their sensory properties, especially acidity. In order to improve their sensory properties they are mixed with

other juices. The most common juice used for this purpose is an apple juice. Considering the lower antioxidative potential of apple juice, the main objective of this work was to investigate the influence of apple juice added in different amount in blackberry and sour cherry juices on their total polyphenols content and antioxidative capacity. The other objective was to evaluate the sensory acceptable amount of small fruit juice in the mixture with apple juice that would still have a significant level of total polyphenols and high antioxidative effect.

MATERIAL AND METHODS

Preparation of juices

Blackberry, sour cherry and apple juices were obtained from concentrated fruit juice, diluted to a dry matter prescribed by regulation (Službeni Glasnik, Republike Srbije, 2011). Blackberry and sour cherry juices were mixture with 0, 20, 40, 60 or 80 % of apple juice.

Total polyphenol content

The total polyphenols content was determined by using the Folin-Ciocalteu method as described in the work of Fu et al., (2011). Two hundred microliters of sample solution diluted in appropriate solvent was mixed with 1000 µl of 1:10 diluted Folin-Ciocalteu reagent. After 6 min, 800 µl of sodium carbonate (75 g/l) was added. The sample stood for 120 min at room temperature before the absorbance was measured at 760 nm. As a control, the solvent was used instead of the diluted sample. Gallic acid was used as a standard, and a total polyphenol content was expressed as a mg gallic acid equivalent (mg GAE)/l of juices. Triplicate measurements were taken and mean values were calculated.

Ferric-reducing antioxidant power (FRAP) assay

The FRAP test is based on the reducing potential of antioxidant substances. This method was used to determine the antioxidant activity of juices. FRAP method was done according to the procedure described in the work of Fu et al. (2011). Briefly, FRAP reagent was made of acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl) and $FeCl_3$ solution (20 mM in water) in a volume ratio of 10:1:1, respectively. A sample containing 3 ml of freshly prepared FRAP and 100 µl of the diluted sample was incubated at 37 °C for 4 min and the absorbance was measured at 593 nm. The standard curve was constructed using $FeSO_4$ solution, and the results were expressed as µmol Fe (II)/ l of juices. All test analyses were run in triplicate.

Statistical analysis

All measurements were done in triplicate and results were expressed as mean ± standard deviation. The results of total polyphenol content and antioxidant capacity (FRAP method) were analysed using ANOVA and Tuckey's HSD test.

RESULTS AND DISCUSSION

Small fruit juices such as blackberry and sour cherry juices, could not be consumed in large quantities because of its high acidity. In order to reduce their acidity the apple juice at different proportion was added. The aim of this study was to evaluate how the addition of apple juice (0, 20, 40, 60 and 80 %) in these acidic juices affected their total phenolics content and antioxidant capacity.

The Folin-Ciocalteu method measures the reduction of the reagent by phenolic compounds with the formation of a blue complex that can be measured at 760 nm against gallic acid as a standard (Imeh & Khokhar, 2002). As it could be expected, the values for total polyphenols gradually decreased in both small fruit juice mixtures, with increasing the proportion of apple juice. The content of total polyphenols in the studied juice samples is shown in Fig. 1. In addition, in 100 % apple juice the total polyphenol content was 522.35 ± 14.73 mg GAE/l of juice. The lowest amount of total phenols in blended juices was determined for a sample with

20 % of sour cherry juice (773,11 mg GAE/l of juice), while the highest amount was in 100 % of sour cherry juice (2485,57 mg GAE/l of juice) (Fig.1).

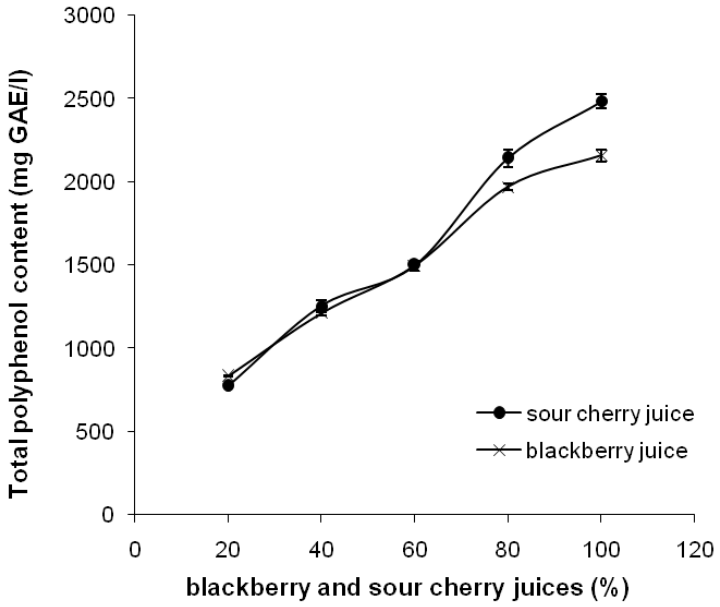

Fig.1. The total phenols content of small fruit juices blended with apple juice

The values obtained for the antioxidant activity with FRAP method also decreased with the addition of apple juices in small fruit juices. The values obtained for antioxidative activity of tested juices by FRAP assay are shown in Fig. 2. In addition, the FRAP value for 100 % apple juice was 4776.43 ± 72.49 µmol Fe (II)/l of juice. The lowest FRAP value of the blended juices was obtained for a sample with 20 % of sour cherry juice (7188.95 µmol Fe (II)/l of juice), while the highest antioxidative activity was performed for 100 % of blackberry juice (30907.14 µmol Fe (II)/l of juice) (Fig. 2).

Generally, the values of antioxidative activity were higher in sour cherry mixtures than in blackberry mixtures, while the value of total polyphenols were similar in both studied juice mixtures until the 60 % of small fruit juice content. Further increase in the proportion of small fruit juices to 80 and 100 % resulted in higher phenolic contents in mixtures with sour cherry juices in relation to blackberry juices. Also, comparing these results with literature, similar values were reported for these two methods in a work of Fu et al., (2011); Piljac-Žegarac & Šamec, (2011); Müller et al., (2010); Piljac-Žegarac et al., (2009); Bermudez-Soto & Tomas-Barberan, (2004); and Deighton et al., (2000).

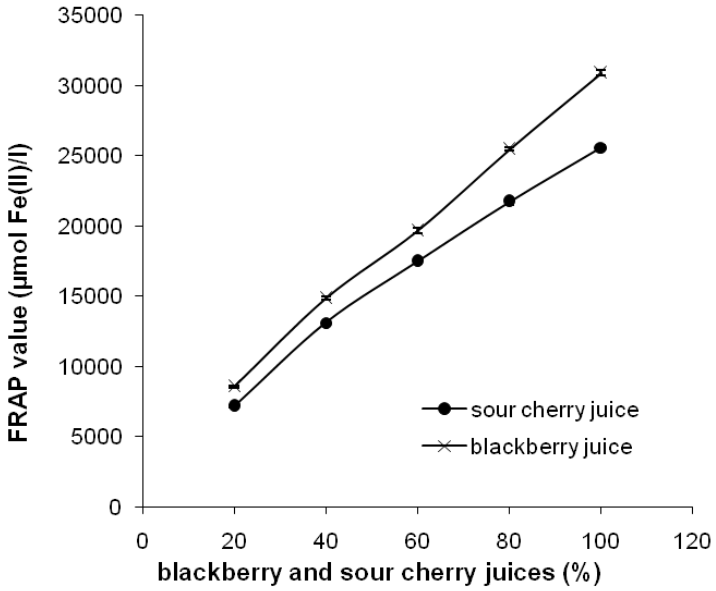

Fig. 2. The antioxidative activity of blackberry and sour cherry fruit juices blended with apple juice

The correlation between total polyphenol content and antioxidant activity is presented in Fig. 3.

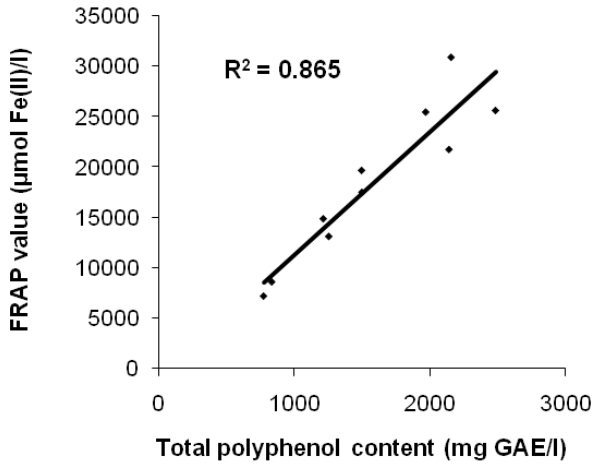

Fig. 3. Correlation between the antioxidant capacities and total phenol content

A highly positive correlation (R^2= 0.864) between the FRAP value and total phenolic content indicated that phenolic compounds could be one of the main components responsible for antioxidative capacity of these juices. A similar value for the correlation coefficient between these two methods was obtained in the work of Fu et al. (2011). However, the better correlation coefficient was performed in the work of Deighton et al. (2000).

Preliminary sensory analysis showed that the addition of apple juice in blackberry and sour cherry juices was necessary in a certain amount. The best evaluated samples were equally the mixture with 40 and 60 % of either blackberry or sour cherry juices (data not shown). This means that the reduction of total polyphenol content and antioxidant capacity was approximately 50 % with the addition of apple juice in best-rated samples. However, these results suggest that in the production of small fruit juices the amount of small fruit juice in the mixture with the apple juice could be even 60 %. The juices with such a high proportion of small fruit would certainly represent important sources of polyphenols in our diet and could be regarded as functional food.

CONCLUSIONS

Blackberries and sour cherries are widespread in our country and present a rich sources of flavonoid and other polyphenols. It should be emphasized that the consumption of small fruit juices is highly desirable due to its strong antioxidative capacity.The results obtained in this study showed that even though the addition of apple juice reduced the antioxidant potential in blackberry and sour cherry juices, the best sensory evaluated samples still had a high level of total polyphenols and high antioxidant capacity. Also, these results support the previous findings that small fruits and their products possess potent antioxidant effects and make foundation for further research in order to enhance health benefits of small fruit.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education and Science, Republic of Serbia (Grant No. 46001).

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LYCOPENE CONTENT AND ANTIOXIDANT CAPACITY OF TOMATO JAM

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ABSTRACT: Tomato (*Solanum lycopersicum*) is the world's second largest vegetable crop after the potato and a major source of lycopene. Lycopene is a red carotenoid and an efficient free radical scavenger, which presence in the diet positively correlates with reduced risk of chronic diseases. Although tomatoes are usually used as a salad vegetable, more than 80 % of tomatoes consumption comes from processed products such as ketchup, tomato juice, pickled tomatoes, sauces etc. Jam is another possibility of tomato processing. Tomato jam was produced in an open 120 liters oil jacketed stainless steel kettle. The proportion of sugar to fruit was 1:2.5, while the final concentration was 65°Brix (Bx). Lycopene content was estimated spectophotometrically at 503 nm and concentration was calculated using molar extinction coefficient of 17.2 x 10^4 $M^{-1}cm^{-1}$. An examination of the antioxidant capacity of samples was performed using DPPH assay. Obtained results indicate that lycopene content of tomato jam was slightly lower compared to fresh ones (79.66 mg/kg in fresh tomatoes and 75.54 mg/kg in tomato jam), while antioxidant capacity has increased during processing (0.335 and 1.195 mM Trolox equivalents per kilogram of samples for fresh tomatoes and tomato jam, respectively). Moreover, heat processing of tomatoes could result in increased bioavailability of lycopene. Based on the results, it may be concluded that tomato jam can be a high-quality product which can be a desirable component of healthy diet.
Key words: *tomato jam, lycopene, antioxidant capacity*

INTRODUCTION

Tomato (*Solanum lycopersicum*) is the world's second largest vegetable crop after potato, with more than 152 million tons produced in 2010 (http://faostat.fao.org). It is native to the Andes of South America and evolved from the cherry tomato (Jy et al., 2004). The world consumption of tomato is increasing, but varies greatly between countries and regions (Slimestad and Verheul, 2009). Although tomatoes are usually used as a salad vegetable, more than 80 % of tomatoes consumption comes from processed products such as ketchup, tomato juice, pickled tomatoes, sauces, paste, purée, etc. (Gould, 1992). Tomato is a very important component of healthy diet and rich source of natural bioactive compounds. Numerous studies have shown that increased consumption of tomato and tomato products has been linked with decreased risk of various chronic diseases, such as cardiovascular and cancer diseases (Heber and Lu, 2002). Compound which may be responsible for such effects of tomato is lycopene. However, many other compounds (polyphenols, vitamins, minerals) can contribute to beneficial effects of tomato consumption. The nutritional value of tomatoes varies with the stage of maturity, with the highest concentration of bioactive compounds in fully ripe tomatoes (Motamedzadegan and Shahiri Tabarestani, 2011).
Lycopene is a red colored acyclic carotenoid without provitamin A activity, which contains 11 conjugated double bonds arranged linearly in the all-*trans* form (Stahl and Sies, 1996). In addition to conjugated, lycopene contain and 2 unconjugated double bonds, but do not have the terminal β-ionic ring. The main dietary sources of lycopene are tomato and tomato-based products (more than 80 %), but it is also found in moderate to high concentrations in some other fruits and vegetables, such as watermelon, red grapefruit, apricots, guava and papaya (Clinton, 1998). The lycopene content of tomato varies significantly with ripening and the variety of tomatoes and is linked with the content of insoluble solids (Sharma and Le Maguer, 1996; Thompson et al., 2000).

The first investigations on bioactive effects of lycopene were shown that intraperitoneally injected lycopene increases the survival rate of X-irradiated mice and the resistance of mice toward bacterial infections (Forssberg et al., 1959; Lingen et al., 1959). After these first studies, numerous of epidemiological and clinical researches indicate protective effects of lycopene on various diseases, e.g., cardiovascular diseases, prostate, gastrointestinal and epithelial cell cancer, osteoporosis, cataract etc. (Rao and Rao, 2007). Biological activities of lycopene include antioxidant activities (singlet oxygen quenching, scavenging of peroxyl radicals), induction of cell-cell communication and growth control (Martinez-Valverde et al., 2002). Lycopene is one of the most efficient natural antioxidants, probably due to the presence of a long chain of conjugated double bonds. However, the increased reactivity of lycopene compared to other carotenoids is related to the presence of the two nonconjugated double bonds (Conn et al., 1991). All-*trans* isomeric form of lycopene is primarily present in nature, but it can undergo mono or poly isomerization during tomato processing and storage by light, thermal energy and chemical reactions to its *cis*-isomeric form. The formation of *cis* isomers may decrease biological activity of lycopene (Lee and Chen, 2002). Lycopene is a relatively stable under the conditions of thermal processing, but its stability varies in different food systems due to the complex nature of food components (Agarwal et al., 2001). Some studies indicate that uptake of lycopene is greater from heat processed tomato products than from raw tomato (Rao and Agarwal, 1998).

In addition to lycopene, tomatoes contain many other valuable components which can contribute to a healthy diet. These compounds include flavonoids and phenolic acids, ascorbic acid, vitamin E and carotenoids. Polyphenols are very efficient natural antioxidants and are associated with reduced risk of cardiovascular diseases and some type of cancer. The main phenolic compounds of tomato are quercetin, naringenin, rutin and chlorogenic acid (Slimestad and Verheul, 2009).

The main objective of this study was to determine the lycopene content and antioxidant properties of tomato jam and raw tomato used in jam production.

MATERIAL AND METHODS

Tomato used in jam production was obtained from experimental school estate “Radmilovac” of Faculty of Agriculture, Belgrade. Hexane, butylated hydroxytoluene, sodium acetate, glacial acetic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone was obtained from Fisher Scientific (Loughborough, United Kingdom).

Jam production

Tomato jam was produced in an open 120 liters oil jacketed stainless steel kettle. After sorting, the tomatoes were washed in cold water and clean and sound tomatoes were crushed. The obtained tomato pulp (50 liters) with 4 % w/w of soluble dry matter was blended with ascorbic acid (prevents oxidation) and one-half of the total sugar to be added (10 kg). The mixture was heated to 90°C with mechanical stirring (40 rpm) to evaporate part of the water. When the mixture was well homogenized and soluble solids content (Brix) was significantly increased, the rest of the sugar was added to reach the total sugar level of 65°Brix. At the same time, the pH was adjusted to 3.3 ± 0.1 with a citric acid solution (10 % w/v) and required amount of pectin solution (5 % w/v solution of high methoxyl pectin HM 64020 in water) was then added. Afterwards, the temperature was raised to 95°C in order to achieve final soluble solids content and enable the filling of hot jam without subsequent sterilization. When the finished point at 65°Brix and the appropriate consistency was reached, the hot jam was poured into clean transparent glass jars. The samples were stored in dark place at room temperature. Dry matter of samples was determined using an Abbe refractometer (Bellinghan & Stanley Ltd., United Kingdom).

Lycopene determination

Total lycopene content was determined spectrophotometrically according to method described by Perkins-Veazie *et al*. (2001). A 1 g of samples (tomato jam or fresh tomato) was added to a mixture consisting of 25 ml of hexane, 12.5 ml of acetone, 12.5 ml of ethanol and 0.05 % (w/v) butylated hydroxytoluene. The mixture was stoppered and placed on an orbital shaker to mix at 180 rpm for 15 minutes (temperature of mixing was 5°C). After shaking, 7.5 ml of cold deionized water was added and the mixture was agitated for another 5 min. The suspension was left at room temperature for 10 minutes to allow separation of polar and non-polar layers. The absorbance of non-polar (upper) layer was measured in a 1 cm path length glass cuvette at 503 nm versus a blank of hexane solvent using Jenway 6105 UV/Vis spectrophotometer (Jenway, United Kingdom). The lycopene content was calculated using following equation:

$$\text{Lycopene content (mg/kg)} = \frac{}{}$$

where the molar extinction coefficient of 17.2 x 10^4/Mcm is that reported by Zechmeister et al. (1943).

Determination of antioxidant capacity - DPPH assay

The total antioxidant capacity of samples was determined by DPPH assay using the modified procedure described by Kaneda et al. (1995). For analysis of antioxidants, approximately 10 g of samples was homogenized and dissolved in 30 ml of ethanol solution (70 % v/v). The samples were mixed for 10 minutes at 5°C and then centrifuged at 9000 rpm for 10 minutes. The supernatant was poured off and pellet was re-extracted with 15 ml of ethanol solution by the same procedure. The obtained supernatants were combined and the total volume was adjusted to 50 ml with 70 % v/v ethanol solution.

The extracts of samples (0.2 mL) were added to the DPPH solution (2.8 mL) (mixture of 1.86 x 10^{-4} mol/L DPPH in ethanol and 0.1 M acetate buffer (pH 4.3) in ratio 2:1) and mixed vigorously. After 60 minutes of incubation in a dark place, the absorbance was measured at 525 nm. The standard curve was constructed using 1 mM Trolox solution and the results were expressed as mM Trolox equivalents (TE) per kilogram of sample (tomato or tomato jam).

Statistical analysis

All measurements were conducted in triplicate and data were expressed as mean ± standard deviation. The significance of differences among means was tested using t-test for independent samples.

RESULTS AND DISCUSSION

Tomato and tomato based products are the main dietary source of lycopene, one of the strongest natural antioxidants. Dry matter, lycopene content and antioxidant activity of tomato jam and raw tomato used in its production are presented in table 1. Lycopene content of fresh tomato used in this study are slightly higher than those reported by Martinez *et al.* (18.6-63.37 mg/kg fresh weight) and Clinton (8.8-42.0 mg/kg fresh weight), but similar to those of Nguyen and Schwartz (31-77 mg/kg fresh weight).

Table 1. Dry matter, lycopene content and antioxidant capacity of raw tomato and tomato jam

Samples	Dry matter (%)	Lycopene (mg/kg fresh weight)	Lycopene (mg/kg dry matter)	DPPH (mM TE/kg sample)
Raw tomato	4.0	79.66 ± 1.14	1991.50 ± 28.56	0.335 ± 0.008
Tomato jam	65.0	75.54 ± 1.38	908.91 ± 16.59	1.195 ± 0.059

Values of lycopene content and antioxidant activity are means of three determinations ± standard deviation

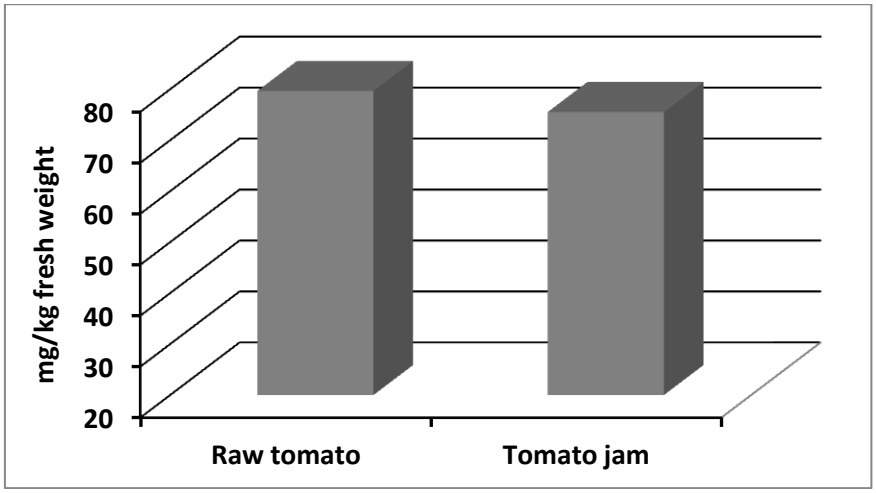

Figure 1. Lycopene content of raw tomato and tomato jam

Tomato jam has statistically significantly lower content of lycopene ($p = 0.018$; $p<0.05$) compared to fresh tomato used in its production. However, the lycopene content of tomato jam was lower by only 4.12 mg/kg in comparison with fresh tomato. The lycopene content expressed as mg/kg dry matter was more than twice lower than in fresh tomato. Given that 8.31 % of tomato jam dry matter was originating from tomato, the absolute content of lycopene was reduced by 54.34 % during the jam production.

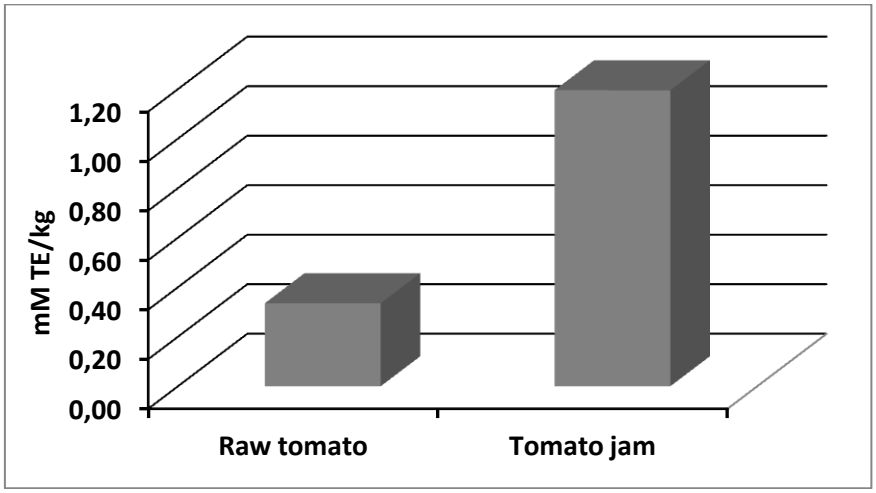

Figure 2. Antioxidant capacity of raw tomato and tomato jam

Antioxidant capacity of tomato jam was very significantly higher compared to raw tomato (more than 3.5 times). This result is in accordance with those found in literature where Wang *et al.* (1996) and Takeoka *et al.* (2001) have reported that heat-processed tomato juice and

tomato paste had a much higher antioxidant capacity than fresh tomato. Such increases in antioxidant capacity as a result of heat processing are still not completely explained.
In addition, tomato jam was characterized by typical red-brown color with low granulous texture and shiny surface, enjoyable fruit taste and intense pleasant fragrance (internal laboratory sensory evaluation).

CONCLUSIONS

Results showed that the lycopene content of tomato jam was slightly, but significantly lower than in fresh tomato. In the other hand, the absolute lycopene content was reduced more than 50 % during thermal processing of tomatoes into jam. However, many studies have shown that uptake of lycopene is greater from heated-processed tomato products than from unprocessed tomato. The reason for this occurrence is probably enhanced bioavailability due to breaking down of sturdy cell walls, thus making lycopene more accessible. Antioxidant capacity of tomato jam was more than 3.5 times greater in comparison with raw tomato. In addition, tomato jam had pleasant sensory profile with retained distinctive tomato aroma. Obtained results suggest that tomato jam can be a high-quality product which can be a desirable component of healthy diet.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education and Science, grant No. 46001.

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ANTIOXIDANT AND ANTIMICROBIAL POTENTIALS OF CHAMPIGNON MUSHROOM

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ABSTRACT: Fruiting bodies of some wild and cultivatable mushrooms contain medicinal compounds which are being used in traditional medicines and cosmetics. Champignon mushroom (*Agaricus bisporus*) is the most widely cultivated species of edible mushroom worldwide. This paper focuses on antioxidant and antimicrobial importance of *A. bisporus*. Water-soluble polysaccharide-enriched fraction was isolated from the dry carpophores of *Agaricus bisporus*. Antioxidant activities were investigated using *in vitro* assay systems: 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and chelating ability on ferrous ions. Antimicrobial activity was tested against Gram positive and Gram negative bacteria *in vitro* by disk diffusion method in order to determine the zones of inhibition. At concentrations of 0.1-10 mg/ml, the scavenging abilities of *A. bisporus* ranged between 12.3-75.5 %. The radical scavenging ability of the positive controls -tocopherol and ascorbic acid, at the concentrations of 0.1-20 mg/ml, were between 79.9-80.8 and 80.6-91.1 %, respectively. Polysaccharide extract from *A. bisporus* showed steadily increasing chelating ability as concentrations increased to 88.2 % at 20 mg/ml. The chelating ability of the citric acid was between 7.2-10.7 %, at the concentrations of 0.1-20 mg/ml. The study of antimicrobial potential of polysaccharide extract showed more potent activity against Gram-positive *Enterococcus faecalis* ATCC 49532 (26.7 ± 0.2 mm), *Bacillus cereus* 10876 (27.5 ± 0.4 mm), *Geobacillus stearothermophylus* ATCC 7953 (22.8 ± 0.3 mm) than Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 35032 (10.4 ± 0.6 mm), *Proteus hauseri* ATCC 13315 (12.1 ± 0.1 mm) *Escherichia coli* (0157:H7) 35150 (12.7 ± 0.4 mm) with exception of *Klebsiella pneumoniae* ATCC 27736 (22.3 ± 0.2 mm).
Key words: *Agaricus bisporus, antioxidant, antimicrobial, polysaccharide extract*

INTRODUCTION

Agaricus bisporus (J. Lge) Imbach also called champignon, white mushroom, common mushroom or button mushroom is one of the most well-known, most cultivated and most used edible mushrooms. Human beings have been consuming champignons since Ancient times. Ancient Egyptians believed that the *Agaricus bisporus* held the key to immortality, while Ancient Romans revered the mushroom as one of the foods of the gods. During the 1600s, the French began to cultivate *Agaricus bisporus*, using dark underground tunnels beneath Paris that are still used for mushroom growing today (Spencer, 1985). *Agaricus bisporus* is now cultivated in at least 70 countries around the world (Cappelli, 1984). Global production in the early 1990s was reported to be more than 1.5 billion kg, worth more than US$ 2 billion (Chang, 1993).

In addition to its own unique flavor, eating this mushroom may provide important health and nutrition benefits when made a regular part of the diet. *Agaricus bisporus* is a source of excellent nutrition, providing a range of vitamins, minerals, carbohydrates, protein and phytochemicals that are important for human health. It provide the minerals selenium, copper, potassium, iron and zinc, as well as a range of vitamins including thiamin, riboflavin, pantothenic acid, niacin and vitamins C and D (Chang, 1993; Spencer, 1985).

In addition to the nutritional benefits of this mushroom, it may have useful medicinal properties that support health and well-being. *Agaricus bisporus* significantly stimulated

immune activity, specifically cytokines and enzymes that are responsible for inflammation (Kozarski et al., 2011; Ren et al., 2008; Wu et al., 2007). *Agaricus bisporus* may prevent breast cancer through an aromatase-inhibiting action that reduces enzymes that increase estrogen levels and drive breast cancer growth, making the mushroom both hormone-balancing and chemo-preventative (Chen et al., 2006; Grube et al., 2001).
Recently, there has been growing interest for polysaccharides of mushrooms, since there has been reported a great influence of these components on human health. Polysaccharides are potentially useful biologically active ingredients for pharmaceutical use, such as for immune regulation, for anti-radiation, anti-blood coagulation, anti-cancer, anti-HIV and hypoglycemic activities (Klaus et al., 2009; Kozarski et al., 2009; Yang et al., 2005; Yoon et al., 2003; Lee et al., 2002). The mushroom-derived polysaccharides lentinan, schizophyllan, and krestin have been accepted as immunoceuticals in Japan, Korea and China (Zheng et al., 2005). The activity of polysaccharides is determined by their conformation, composition and size (Bohn and BeMiller, 1995).
In the present study, we have determined the antioxidant activity of polysaccharide enriched water soluble extract from *Agaricus bisporus* using assays pertaining to different ways of antioxidant action. Antimicrobial potential of polysaccharide extract was observed on several microorganisms of medicinal importance.

MATERIAL AND METHODS

A. bisporus Horst U1 fruiting bodies were obtained from the Mushroom Experimental station (Horst, The Netherlands). Crude polysaccharide extracts were prepared by hot water extraction as described before (Klaus et al., 2011). Polysaccharides were semi-purified by precipitation in 65% ethanol and repeated washing to remove the excess mannitol and ethanol-soluble phenolic compounds. The preciptitate was dried at 42 °C, in vacuum and stored for further use. The yield of extract was 3.7 ± 0.2 g/100g express on a dry weight basis of fruiting body.

Antioxidant activity assays

Antioxidant activities were investigated using *in vitro* assay systems: 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capability and chelating ability on ferrous ions.

Scavenging capability for DPPH radicals

The assay was done according to the modified method of Ekanayake et al, (2005). In the first series the 2 ml of extract dissolved in dimethyl sulfoxide (DMSO) was mixed with 1 ml freshly prepared DMSO solution of 0.2 mM DPPH. In the second series, extract (2 ml) was mixed with 1 ml DMSO solution. Both series were placed in the dark at room temperature for 1 hour. The radical-scavenging activity was calculated as a percentage of DPPH discoloration using the equation: $[1-(A_i-A_j)/A_c]$ x100, where A_i was the absorbance of 2 ml extract mixed with 1 ml DPPH solution, A_j was the absorbance of 2 ml extract mixed with 1 ml DMSO solution and A_c was the absorbance of blank-2 ml of DMSO mixed with 1 ml of DPPH solution. Ascorbic acid, butylated hydroxytoluene (BHT) and α-tocopherol dissolved in DMSO were used as the positive control. The EC_{50} value (mg extract/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis**.** EC_{50} was calculated concerning concentration of 5 mg/ml at which extract reached maximum in DPPH radical scavenging activity.

Chelating ability on ferrous ions

Polysaccharide powder (0.1 to 20 mg/ml, 1 ml) in Milli-Q water was mixed with 3.7 ml of methanol and tested for Fe^{2+} chelating ability according to the method of Dinis et al., (1994). The Fe^{2+} was monitored by measuring the formation of ferrous iron-ferrozine complex at 562 nm. The lower the absorbance of the reaction mixture, the higher the Fe^{2+}-chelating ability. The EC_{50} value (mg extract/ml) is the effective concentration at which ferrous ions were chelated by 50%. Citric acid and ethylenediaminetetraacetic acid (EDTA) were used for comparison.

Antimicrobial activity

Microorganisms
Antimicrobial activities of the crude polysaccharide extract of *A. bisporus* on Gram-positive *Enterococcus faecalis* ATCC 49532, *Bacillus cereus* 10876, *Geobacillus stearothermophylus* ATCC 7953 and Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 35032, *Proteus hauseri* ATCC 13315, *Escherichia coli* (0157:H7) 35150 and *Klebsiella pneumoniae* ATCC 27736 were investigated in this study. The bacteria were obtained from the culture collection of the Department for Industrial Microbiology, University of Belgrade - Faculty of Agriculture. All bacterial strains were stored at +4^{o}C on appropriate agar slants, subcultured every two weeks and checked for purity.

Screening of antimicrobial activity of extract by disc-diffusion test
The disk-diffusion method was applied for the evaluation of the antibacterial activities of the polysaccharide extract. The bacterial cells were washed from the surface of agar and suspended in sterile saline to a concentration of 1.0x10^{5} CFU/ml. Appropriate agar in Petri dish was seeded with 100 µl of bacterial suspension. The crude polysaccharide extract was dissolved in DMSO to a final concentration of 30 mg/ml and filter-sterilized through a 0.22 µm membrane filter. On the surface of the agar, the 6 mm filter discs were placed (three discs per agar plate). Ten microliters of the tested crude polysaccharide extract were added to the disc. Reference discs used for control contained penicillin and tetracycline. The plates with bacterial cultures were incubated overnight at 37^{o}C and the diameter of the resulting zone of inhibition was measured and compared with those of reference discs. Inhibitory activity of DMSO was also tested.

RESULTS AND DISCUSSION

The results of scavenging ability and chelating ability on ferrous ions of polysaccharide extract from of *A. bisporus* are shown on figure 1 and 2. As it can be seen, at concentrations of 0.1-10 mg/ml, the scavenging abilities of *A. bisporus* polisaccharide enriched extract on DPPH radicals were between 12.3-75.5% (Figure 1). At 0.1-20 mg/ml, the radical scavenging ability of the positive controls BHT, ascorbic acid and α-tocopherol were between 1.1-69.1, 80.6-91.1 and 79.9-80.8 %, respectively. The radical scavenging ability at of the extracts and positive controls, at 5 mg/ml decreased in the following order: ascorbic acid > α-tocopherol ≈ *A. bisporus* > BHT. EC_{50} values of the DPPH scavenging activity of the polysaccharides from champignon mushroom was 2.0 ± 0.18 mg/ml.

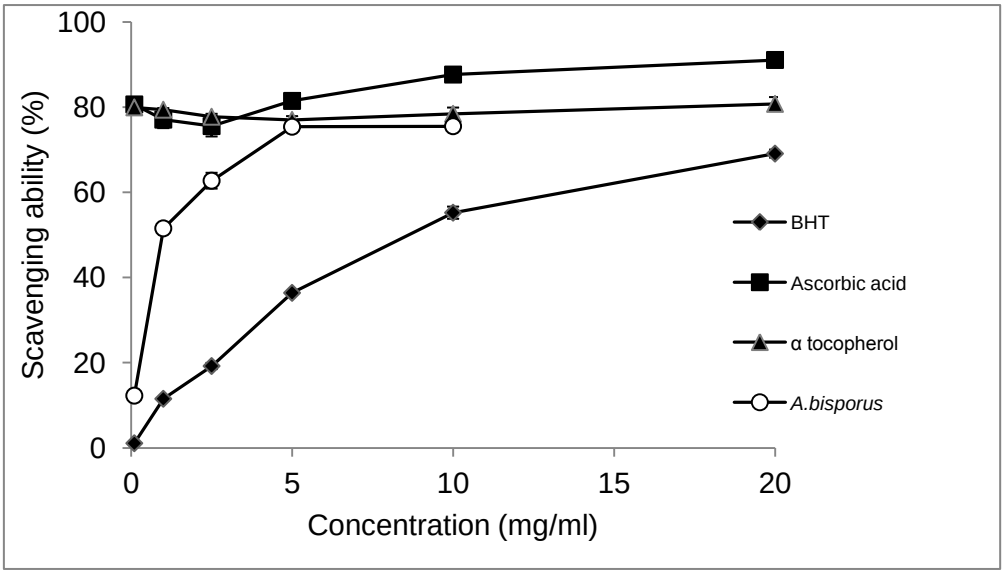

Figure 1. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals of polysaccharide- enriched extract from of *A. bisporus*. Each value is expressed as mean ± standard deviation (*n* = 3).

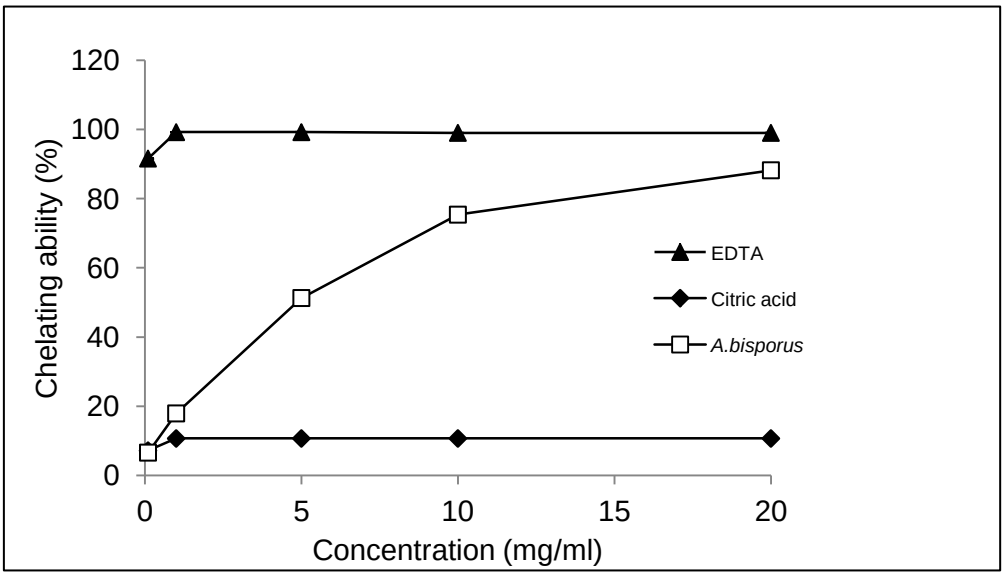

Figure 2. Chelating ability on ferrous ions of polysaccharide-enriched extract from of *A. bisporus*. Each value is expressed as mean ± standard deviation (*n* = 3).

Chelating effects of the polysaccharide extract from *A. bisporus* on ferrous ion increased with the increased concentrations (Figure 2). At 0.1-20 mg/ml, the chelating ability of *A. bisporus* polysaccharide extract was between 6.6-88.2%. The chelating effect of the synthetic metal chelator EDTA was between 91.6-99% at 0.1-20 mg/ml, while citric acid was not as good chelating agent for ferrous ions in this assay and its chelating ability was 10.7% at 20 mg/ml. EC_{50} values of the chelating ability of ferrous ions for *A.bisporus* extract was 7.80 ± 0.21 mg/ml.

The main mechanism of ferrous ion chelating activity is the ability of chelators to deactivate and/or chelate Fe^{2+} which can promote the Fenton reaction and hydroperoxide decomposition. Iron toxicity (high organ storage of iron) and/or high blood levels of iron are associated with an increased risk of free radical damage and cancer. The capability of iron to generate free radicals from peroxides by Fenton reactions has been implicated in

cardiovascular disease. Free radicals formed as a result of high iron can attack low-density lipoproteins (LDL) and subsequently lead to fatty plaque buildup, damage to the walls of arteries, as well as to heart muscle tissue. Chelation therapy may possibly reduce iron-related free radical damage and increase overall survival in cardiovascular disease (Halliwell and Gutteridge, 1990).
Factors affecting and/or attributing to chelating effects of polysaccharide-enriched extract from *A. bisporus* need to be further studied.

Antimicrobial activity of extract
As summarized in Table 1. tested Gram positive bacteria seemed to be more sensitive than Gram negative bacterial strains to the examined polysaccharide extract of *A. bisporus*. The most susceptible bacterium was *Enterococcus faecalis* ATCC 49532 (26.7 ± 0.2). Regarding the fact that all examined bacteria are serious pathogens, their sensitivity to the mushroom extract is of particular interest.

Table 1. Antibacterial acivity (inhibition zone measured in mm, including 6 mm filter discs) of the polysaccharide extract of *A. bisporus*

bacterial strain	***A. bisporus***	**P**	**T**
Enterococcus faecalis ATCC 49532	26.7 ± 0.2	12.6 ± 0.5	24.3 ± 0.6
Bacillus cereus ATCC 10876	27.5 ± 0.4	-	12.4 ± 0.6
Geobacillus stearothermophylus ATCC 7953	22.8 ± 0.3	-	17.6 ± 0.5
Pseudomonas aeruginosa ATCC 35032	10.4 ± 0.6	-	-
Proteus hauseri ATCC 13315	12.1 ± 0.1	19.8 ± 0.2	24.8 ± 0.7
Escherichia coli (0157:H7) ATCC 35150	12.7 ± 0.4	11.8 ± 0.3	13.2 ± 0.6
Klebsiella pneumoniae ATCC 27736	22.3 ± 0.2	15.4 ± 0.3	16.7 ± 0.1

P, penicillin; T, tetracycline; (–) no inhibition.
Each value is expressed as mean ± standard deviation (n = 3).

CONCLUSIONS

Measurements of antioxidant and antimicrobial properties of hot water polysaccharide extract of fruiting bodies of *A. bisporus* showed moderate antioxidant and strong antimicrobial activities. The antioxidant and antimicrobial compounds of the polysaccharide extract were resistant to high temperatures, even to a period of 45 min of boiling at 120ºC during the extraction process.
Champignon mushroom polysaccharides act as natural antioxidant. As oxidative stress appears to be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied.
In recent decades microorganisms are becoming resistant to antibiotics due to their excessive use in medicine, but in food industry, too. On the other hand, it is known that antibiotics can have adverse effects on the health. This study has shown that commonly used mushroom *A. bisporus* could be suitable as food preservative against food spoilage microorganisms, i.e. as antimicrobial agent in the food industry. Further investigations are necessary to verify these activities *in vivo.*

ACKNOWLEDGEMENTS

This paper is a result of the research within the projects III 46001, III 46010 and III 43004, financed by the Ministry of Science and Technological Development, Republic of Serbia. *A. bisporus* fruiting bodies were kindly provided by prof. dr Leo van Griensven, University of Wageningen.

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ANTIOXIDANT PROPERTIES OF SELECTED FIG (*FICUS CARICA* CV. SABZ) FROM IRAN

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ABSTRACT: Fig has been a typical fruit component of the health-promoting in the Middle East for a very long time. Phenolics are an important constituent of fruit quality because of their contribution to the taste, color and nutritional properties of fruit. In this study, the phenolics of fig (*Ficus carica* cv. Sabz) pulp from Iran, stahban region-southern part of Shiraz-were extracted by three different solvents (boiling water, ethanol/water (v:v, 50:50) and methanol/water (v:v,50:50)) in different concentration by ultrasound-assisted method. The total fig phenolics extract (TFPE) was expressed on a fresh weight (FW) basis as mg of gallic acid equivalence (GAE) per 1 kg of fresh weight(FW) .With the HPLC system. The following phenolics were determined: gallic acid, chlorogenic acid, catechin and rutin. Results showed that the highest content among phenolics was 4768.46 (mg GAE/kg FW) related to extracted solution by boiling water solvent with 40%(w:v).The fig pulp is rich in gallic acid, chlorogenic acid, catechin and rutin with maximum amounts of 644,1449.3,621.3 and 978 (mg/kg FW).It is a clear relation between concentration of fig pulp and phenolics content. This special cultivar contains highest level of phenolics, compared with common figs from Mediterranean region.
Key words: *fig (*Ficus carica cv. *Sabz), ultrasound-assisted method, total fig phenolics extract, HPLC*

INTRODUCTION

Fig (*Ficus carica*) is one of the earliest cultivated fruit trees (Solomon et al.,2006).They are a widespread species commonly grown, especially in warm, dry climates. The world production of figs is about one million tons, and it is mostly concentrated in the Mediterranean (Veberic, 2008).*Ficus carica* cv. Sabz is generally produced in Stahban region –southern part of Shiraz- from Iran. Nowadays over than 20000 tons of this cultivar produced in Stahban and Iran become the biggest producer of this specific cultivar. The diameter of *Ficus carica* cv. Sabz is between 33-37 millimetres, its colour is yellow-greenish and dried from the top of fruit before harvested (Faghih, Sabet Sarvestani, 2002).Dried figs contain one of the highest concentrations of phenolics among the commonly consumed fruits and beverages (Vinson et al., 2005).Figs are also an excellent source of minerals, vitamins and dietary fibre; they are fat and cholesterol free and contain a high number of amino acids (Slavin, 2006).

The interest in phenolic compounds has increased over the last few decades, as these agents have become popular among the public, who attribute them several medicinal properties, mostly related to their antioxidant activity (Aviram et al., 2004). Phenolic compounds may serve this purpose by reducing or donating hydrogen to other compounds, scavenging free radicals, and quenching singlet oxygen (Merken and Beecher, 2000; Fattouch et al., 2007; Costa et al., 2009). The content level of phenolics is usually influenced by cultivar (Veberic et al., 2005).Although figs are an important fresh fruit variety in many countries. There are only a few reports dealing with the phenolic contents of these fruit (Solomon and Vaya, 2010; Caliskan and Polat, 2011; Del Caro and Piga, 2008).

The main objective in this study was to report phenolic extraction from *Ficus carica* cv. Sabz grown in Stahban region, southern part of Shiraz. To our knowledge there is no report in this regard. Thus, the aim of this study was to extract polyphenols in different concentrations (w/v,g/ml) of these cultivar using ultrasound assisted method with three different solvents (boiling water, methanol/water (50:50,v:v) and ethanol/water(50:50,v:v)) and give information on the amounts of total phenolic contents by Folin Ciocalteau method as well as some of

phenolic important classes:Gallic acid,Chlorogenic acid,Rutin hydrate and Catechin analyze with HPLC.

MATERIAL AND METHODS

Chemicals

Figs (*Ficus carica* cv. Sabz) at commercial stage of maturity with homogeneous size were packaged in cardboard boxes (in monolayer) and transported to the laboratory for further analyses. Methanol, ethanol and Folin Ciocalteau reagent were purchased from MERCK. Phosphoric acid, gallic acid, chlorogenic acid, rutin and catechin were purchased from Fluka.Sodium carbonate was purchased from Sharlue.

Extraction method

The pulp of 10-15 fig fruits were homogenized using a woring-blender.Four concentrations was determined: 3.3%, 10%, 25% and 40% (w/v, g/ml).Boling water, methanol/water (50:50, v: v) and ethanol/water (50:50, v: v) were three different solvents for the primary extracion.Incubation was done in static mode in different times. For boiling water the incubation times were 15, 30 and 45 minutes and for two others were 5, 15 and 24 hours. The secondary extraction was done by ultrasound probe (SONOPULS ultrasonic homogenizers, BANDELIN Company) for 5 minutes. The mixture of extracted solution with solid pulp of fig was separated by centrifugation in 3000 rpm (Figure 1).

Total phenolics (TP)

TP of each sample were measured according to the Folin Ciocalteau method and results were expressed as mg gallic acid equivalents (GAE) per 1 kg of fresh weight (GAE/1kg FW).1ml of the fig extract was dissolved in 7.75 ml distilled water and mixed with 0.25 ml of Folin Ciocalteau reagent. After 5 min, 1ml of saturated solution of sodium carbonate was added. The mixture had been allowed to stand for 25 min at room temperature and the absorbance was measured at 765 nm using spectrophotometer.

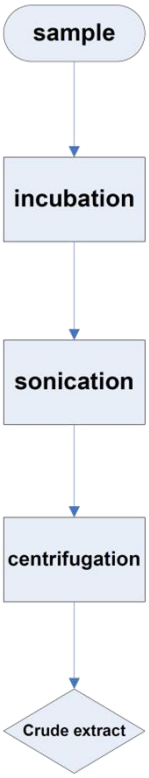

Figure 1.Schematic protocol of ultrasound assisted extraction method

HPLC analysis

The samples were analyzed on the Agilent 1200 series HPLC system with a diode array detector at 280 nm.The elution solvents were aqueous 0.01 M phosphoric acid (A) and 100% methanol (B).The samples were eluted according to the linear gradient described by Escarpa and Gonzalez (1998).The injection amount was 20 µl, and the flow rate was 1 ml/min.The following phenolic compounds were identified: gallic acid, chlorogenic acid, catechin and rutin. Identification of compounds was achieved by comparing the retention times and the spectra as well as by the addition of standards. The concentrations of phenolic compounds were calculated with the help of a corresponding external standard.

RESULTS AND DISCUSSION

Results showed that there is a straight relationship between concentration and total fig phenolics extract (TFPE) (Table 1).As shown, among three solvents, boiling water is the best one for extracting phenolics. In comparison of different samples, concentration of 40% (w/v, g/ml) with 15 min incubation time of boiling water solvent has been the highest amount as a matter of TFPE.

Table 1. Total fig phenolic extracted (TFPE) by (A) Boiling water, (B) ethanol/water (50:50, v: v) and (C) methanol/water (50:50, v: v) in different concentration and incubation time

(A)

Time(min)	Concentration (w/v,g/ml)	TP (mg GAE/1kg FW)
15	3.3% 10% 25% 40%	724.23 2178.84 3239.61 4768.46
30	3.3% 10% 25% 40%	836.92 2885 3155.38 3365.77
45	3.3% 10% 25% 40%	815.77 2423.08 2899.23 4204.23

(B)

Time(h)	Concentration (w/v,g/ml)	TP (mg GAE/1kg FW)
5	3.3% 10% 25% 40%	625.38 1455 2847.69 2640.77
15	3.3% 10% 25% 40%	532.31 1076.92 2475 2133.85
24	3.3% 10% 25% 40%	398.85 1599.61 2930.77 3374.61

(C)

Time(h)	Concentration (w/v,g/ml)	TP(mg GAE/1kg FW)
5	3.3% 10% 25% 40%	449.61 1764.23 2363.46 2955
15	3.3% 10% 25% 40%	171.15 1380 2421.15 2980.77
24	3.3% 10% 25% 40%	301.15 1024.61 2650 2976.54

The maximum amounts of various phenolics for gallic acid is 644 mg/1 kg FW refer to boiling water solvent,40% concentration and 45 min incubation time (Table 2).Other results showed that the highest amount is not just related to specific solvent, but in greater concentration (40% (w/v,g/ml)).

Table 2.Content of various phenolics in fig fruit (mg per 1 kg FW) for (A) boiling water, (B) ethanol/water (50:50, v: v) and (C) methanol/water (50:50, v: v) in different concentration and incubation time

(A)

Concentration (w/v,g/ml)	Incubation time (min)	Gallic acid (mg/1 kg FW)	Catechin (mg/1 kg FW)	Chlorogenic acid (mg/1 kg FW)	Rutin (mg/1 kg FW)
25%	15	296.67	166	367.33	196.67
25%	30	335.33	161.33	326	208.67
25%	45	427.33	169.33	428.67	223.33
40%	15	456.67	240	1449.33	351.33
40%	30	475.33	220.67	465.33	243.33
40%	45	644	112	457.33	270.67

(B)

Concentration (w/v,g/ml)	Incubation time (h)	Gallic acid (mg/1 kg FW)	Catechin (mg/1 kg FW)	Chlorogenic acid (mg/1 kg FW)	Rutin (mg/1 kg FW)
25%	5	207.33	177.33	500	406.67
25%	15	66.67	145.33	629.33	355.33
25%	24	230.67	126.67	528	319.33
40%	5	532	621.33	1088	978
40%	15	312	279.33	797.33	610.67
40%	24	414	476.67	1194.67	580

(C)

Concentration (w/v,g/ml)	Incubation time(h)	Gallic acid (mg/1 kg FW)	Catechin (mg/1 kg FW)	Chlorogenic acid (mg/1 kg FW)	Rutin (mg/1 kg FW)
25%	5	232	199.33	726.67	364.67
25%	15	228.67	200.67	600.67	349.33
25%	24	210.67	160	545.33	316.67
40%	5	366	302.67	946.67	532.67
40%	15	376	172	678.67	476.67
40%	24	378.67	342.67	1129.33	425.33

CONCLUSIONS

To our knowledge, this is the first study extracting and determination total phenolics from *Ficus carica* cv. Sabz. This study showed that considerable amount of antioxidant exist in these especial cultivar. The best solvent for extracting antioxidant is boiling water according to results from Folin Ciocalteau method. With notice to previous research on phenolic capture from different cultivars of fig especially from Mediterranean region, it can be showed that this cultivar is one of the finest cultivars regard to phenolic component. Phenolics extracted from natural sources like figs can be use not only as a medicine application but also for preventing food products from oxidation with low cost level.

ACKNOWLEDGEMENTS

We greatly acknowledge Dr. Mahnaz Ghanbariane for cooperation in HPLC tests.We are grateful for supporting by biological laboratory of chemical engineering faculty of Amirkabir University of Technology.

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COMPOSITION OF HAZELNUTS GROWN IN ALBANIA

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ABSTRACT: Albania is one country with geography on both the Mediterranean moderate climate, hence the possibilities to enlarge the area of nuts cultivation is evident. Scientific study of the hazelnut grown in Albania is important due to the scientific background to the national scheme under implementation for the enlargement of the cultivated area with nuts. Scientific studies on the hazelnut have revealed the nutritional and healthy benefits. Beyond the energetically sources the hazelnut fruit is very important for the nature of the fatty acids which are rich in unsaturated FA.
Preliminary studies on the main constituents from the samples were conducted in two selected sites. The chemical analysis was carried out according to the AOAC (2000) methods. These results are part of an ongoing study started in 2010 and will last for three years. Evidences indicate, that the main constituent in the dried hazelnuts result the triglycerides of 69.1- 70.5 g/100 g dry weight. The carbohydrates vary on 16.2-17.0 g/100 g dry weight hazelnut, and the protein content varies 14.7-15.1 g/100g.
Key words: *Hazelnut,* Corylus Avellana *L., Carbohydrates, Triglycerides, Protein*

INTRODUCTION

Hazelnut (*Corylus avellana L*) has been consumed as fruit for a long time throughout the world, since prehistoric civilization. In the modern times it is used as processed ingredient in the chocolates and other sweets. Main constituents of hazelnuts are fatty acids (FA). The profile is mainly constituted of unsaturated FA, 50 – 73% (Garcia *et al*. 1994). Chemical analyses show that other secondary constituents are phytosterols (Amaral *et al*. 2006a), phenolic compounds and other antioxidants. This compound is supposed to intervene in the controlling other cardiovascular diseases, such as blood hypertension (Alasalvar *et al*. 2006), or in the control of cholesterol level in the blood (Plat and Mensink 2001). Other secondary constituents are vitamins, α-tocopherols (Amaral *et al*. 2006), organic acids and fibers (Botta *et al*. 1994; Alasalvar *et al*. 2006). Recently is reported that hazelnut extracts show antimicrobial activity to the gram-positive bacteria (Oliviera *et al*. 2008).
A number of scientific studies have presented results on the mineral content and vitamins may have indicated by geographical factors (Dunar and Altundag 2004a; Amaral *et al*. 2006 b, c). Studies have confirmed that mineral content in hazelnut is influenced by geography, climate, irrigation and fertilization practices, and the harvesting period in accordance with ripening stage.
Until 1990, the annual domestic production was calculated to reach 100 ton hazelnut and concentrated to the Visoka, Mallakastra region. Recent statistics show that the production have reached 184 ton, in 2011. Mainly it is consumed as unprocessed fruit and in minor part to the confection industry (Rama *et al*. 2011). Actually the consumption is in minimal scale, 0.08 kg/person. The imports are calculated to be 18 ton (DSA, 2007). Compared to the EU countries where the consumption on that assortment is >1kg/person, this value is very low and is a good possibility to ensure a sustainable development to the agriculture to add the cultivation of the *Corylus avellana* L. in the hilly regions of the country.
A very limited data on the chemical composition do exist in the *Corylus avellana L* cultivated in Albania (Osmani-Lataj, Vorpsi and Topi, 2011). Biochemical composition, nutritive values has been the aim of this study. Different regions have been selected for that study and compared to other studies (Koksal *et al*. 2006).

MATERIAL AND METHODS

Plant Material

Seeds of two hazelnut cultivars were harvested during their ripening period 2010-2011, from Visoka plantations, Mallakastra District. The plantation in study was 0.5 ha area, altitude 150m above sea level and Geographic Latitude 40' 36". The samples were stored with husk until conduction of the chemical analysis.

Analytical Methods

Total fat were extracted by Soxhlet at 60^0C per 6 hours and n-hexane was employed as solvent. The hazelnut oil produced was kept in 4^0C in dark place, until GC analysis. The fatty acids were analyzed as Fatty Acid Methyl Esters (FAME) and the equipment employed was GC-FID (Thermo Quest, 2000) with capillary column (23.3m x 0.25mm x 25 μm) according to the AOCS methods (AOCS, 1990). The total ash was calculated according to Koksal *et al*. (2006).

Minerals were analyzed by Spectrophotometer of Atomic Absorption (Varian Spectra AA – 400 Plus). The phosphor was analyzed as phosphomolibdat vanadium according to James (1995) by Spectrophotometer. The chemical analyses were conducted in a four week period after harvesting of the samples.

Statistical analysis

The studied samples was taken during the harvesting period (2010- 2011). Chemical analyses were conducted in three parallel samples from both cultivars. Each sample was of 100 seeds. Data were presented as mean value ±STDEV. Statistical analysis was conducted by Minitab Statistical software (MINITAB INC. 814-238-3280) and standard error $P \leq 0.05$.

RESULTS AND DISCUSSION

Fatty acid composition and total fat

The total fat content was analyzed in both cultivars resulted over 50%, where the *Visoka* cultivar resulted by 61.02% not too much different from *Tonta romana* 60.89%, where identified a number of fatty acids were most important were six of them. Oleic acid was the most abundant by 83.36 %, and others respectively presented in the Table 1. The results on the both cultivars present significant differences for five of FA, except the palmitoleic acid ($P \leq 0.05$) (Table 1). The palmitic acid vary from 4.56% (*Visoka*) to 0.33% (*Tonta romana*), palmitoleic acid 0.33% (*Visoka*) to 0.37% (*Tonto romana*). The oleic acid mean values for both cultivars varied to 83.00%. The linoleic acid content resulted higher to "*Tonta romana*" by 0.42%.

Analysis of minerals

Ash content was significantly high respectively 1.91% (*Visoka*) and 1.97% (*Tonta romana*) a mean value of 1.94% ($P \leq 0.02$) (Table 2). The minerals in higher values resulted potassium by 620.25 mg $100g^{-1}$, phosphor by 251.2 mg $100g^{-1}$ and calcium (113.32 mg $100g^{-1}$). Higher value to the phosphor resulted to *Visoka cultivar by* 290.1 mg $100g^{-1}$ while *Tonta romana* resulted by 212.3 mg $100g^{-1}$. The potassium varied from 112.2 mg $100g^{-1}$ (*Tonta romana*) to 114.44 mg $100g^{-1}$ (*Visoka*). Higher content to the manganese resulted to *Tonta romana by* 63.92 mg $100g^{-1}$. The *Visoka* cultivar resulted with higher content of copper by 1.82 mg $100g^{-1}$, while the *Tonta romana* by 1.14 mg $100g^{-1}$. The iron measured to *Visoka* resulted *to* 3.71 mg $100g^{-1}$ and *Tonta romana* by 2.36 mg $100g^{-1}$. Zinc resulted to 2.09 mg $100g^{-1}$ (*Tonta romana*), and the minimum value 2.05 mg $100g^{-1}$ (*Visoka*).

Table 1: Total fat (g/100g); fatty acids (% FAME) as mean ± STDEV

Fatty acid	Hazelnut cultivars		Mean
	Visoka	Tonta Romana	
Total fat	61.02± 0.27	60.89± 0.29	60.46± 0.28
Palmitic	4.56± 0.15	0.33± 0.15	2.45± 0.15
Palmitoleic	0.33± 0.04	0.37± 0.08	0.35± 0.06
Stearic	1.82± 0.05	1.87± 0.04	1.85± 0.04
Oleic	81.51± 0.11	85.21± 0.46	83.36± 0.28
Linoleic	7.89± 0.23	8.31± 0.02	8.10± 0.12
Linolenic	0.15± 0.04	0.11± 0.02	0.13± 0.03
SFA	6.44± 0.18	7.04± 0.06	6.74± 0.06
PUFA	93.56± 0.32	92.96± 0.1	93.26± 0.16
SFA/PUFA	13.56± 0.44	13.2± 0.12	13.38± 0.26

Comparison of the results with data from the literature indicates similarity in the mineral content with cultivars of Eastern Mediterranean Sea (Alasalvar. et al, 2003; Koksal et al, 2006; USDA, 2007). A recent publication, in the Balkan region, has published that palmitic acid values were higher levels compared to the cultivars of eastern region of Black Sea 4.72 – 5.87% (Köksal et al. 2006).

Table 2: Ash content (g $100g^{-1}$), minerals (mg $100g^{-1}$) expressed as mean value ± STDEV (P≤0.05)

Mineral	Hazelnut cultivars		Mean
	Visoka	Tonta Romana	
Ash	1.91± 0.01	1.97± 0.03	1.94± 0.02
Potassium	680.20± 10.60	560.30± 8.40	620.25±9.50
Phosphor	290.10± 11.3	212.30± 13.5	251.2± 24.4
Calcium	114.44±0.26	112.20± 0.10	113.32± 0.18
Magnesium	61.04± 0.26	63.92± 0.40	62.48± 0.33
Sodium	9.90± 0.40	6.81± 0.47	8.355± 0.43
Manganese	11.04± 0.28	9.68± 0.34	10.36± 0.30
Copper	1.82± 0.08	1.14± 0.04	1.48± 0.06
Iron	3.71± 0.06	2.36± 0.08	3.68± 0.07
Zinc	2.05± 0.02	2.09± 0.04	2.07± 0.03

While the palmitoleic and Stearic acids were in similar values to that of eastern region of Black Sea (Köksal et al. 2006), Stearic acid (2.01%) content resulted higher that our results (1.90%). Oliviera et al. (2008) has reported the mean values of Stearic acid, 1.80% for these cultivars. In our study mean values of the Stearic acid was 1.85% in both cultivars. Oleic acid content 83.36% higher than these of Köksal *et al*., (2006) for cultivars of Black Sea (79.58%). Our results on oleic acid for cultivars *Visoka* and *Tonta romana* differ slightly to these of (Köksal et al. 2006). Mean value of Linoleic acid (8.81%) were lower than those reported in the study (13.0%) of Köksal et al. (2006). Based on the data, the results of FA may attribute mainly to the geography, but the differences on the FA profiles are not so different, significantly. The ash content was significantly different between two cultivars 1.91% (*Visoka*) and 1.97% (*Tonta romana*) ($P \leq 0.02$) (Table 2).

CONCLUSIONS

This study analyses the chemical composition of two hazelnut cultivars in the Mallakastra region. The fat content of the cultivars resulted over 50%, in accordance with results from

other publications (Özdemir et al. 2006). The fatty acid profiles (Table 1) were similar with cultivars of other publications (Garcia et al. 1994.).
Mineral in higher values resulted potassium by 620.25 mg$100g^{-1}$, phosphor by 251.2 mg $100g^{-1}$ and calcium 113.32 mg $100g^{-1}$. Maximum phosphor content was found to *Visoka* by 290.1 mg $100g^{-1}$ and minimum to *Tonta romana* (212.3 mg $100g^{-1}$). The *Visoka* cultivar resulted by maximum iron content (3.71 mg $100g^{-1}$). The maximum zinc content resulted to "*Tonta romana*" cultivar by 2.09 mg $100g^{-1}$. Both cultivars in the study are a good source of bioactive fatty lipids and essential minerals. The essential minerals for both cultivars resulted cooper (1.48%), manganese (10.36%), iron (3.68%), magnesium (62.48%), phosphor (25.12%), zinc (2.07%). These data are comparable to the publications on the literature review. That bring to the result that cultivation of these cultivars in Visoka plantations has significant interest. Further studies are important to the comparison of the results and to have a database that will give a broad panorama related to the other climatic and human factors in the hazelnut cultivation.

ACKNOWLEDGEMENTS

This study was financed by the National Project on Nuts in Albania.

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FOLATE AND CALCIUM INTAKE AMONG UNIVERSITY STUDENT POPULATION IN CROATIAN MOUNTAIN REGION

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ABSTRACT: Inadequate calcium intake may result in weak bones, especially for women who have a greater risk of osteoporosis later in life. This point should be emphasized for young people who need to maximize their peak bone mass, so an ideal time for prevention of osteoporosis may be during the college years. Adequate folate intake in women of childbearing age can reduce the risk of serious birth defects, including neural tube defects. The aim of this study was to determine the average dietary folate and calcium intake among young people from mountain region, taking into account the importance of these two micronutrients in health. In the mountain region, milk and dairy products are traditionally present in a daily diet, which is not the case for the consumption of fruits and vegetables. The study included 321 university students both gender (50.2% females and 49.8% males), 19-25 years old, from Croatian mountain region. Validated food frequency questionnaires were used to assess an average folate and calcium intake. The average dietary folate intake was 239.8 μg DFE i.e. 60.0% DRI. The average dietary calcium intake was 1062.0 mg i.e. 106.2% DRI. The average dietary folate and calcium intake was not significantly different ($p<0.05$) according to gender. As was expected, the average daily calcium intake was in accordance with the recommended value, while the average daily folate intake was below recommended value. Higher intake of folate-rich foods should be advised to university students in Croatian mountain region.
Key words: *folate, calcium, food frequency questionnaire, student population*

INTRODUCTION

The role of folate in normal cell division makes it particularly important in embryogenesis, thus adequate folate intake is important for women of childbearing age because in period before conception can reduce the risk of serious birth defects, including neural tube defects (Lee Gallagher, 2008). To reduce the risk for neural tube defects, it was recommended that women capable of becoming pregnant consume 400 μg of folic acid daily from fortified foods, supplements, or both, in addition to food folate from a varied diet (Picciano et al., 2009). The current Dietary Reference Intakes (DRIs) express folate requirements in terms of Dietary Folate Equivalent (DFEs) to take into account this difference in bioavailability between food folate and synthetic folic acid (Institute of Medicine, 1998). Bioavailability estimates for folate range from approximately 50% for naturally occurring food folate to 100% for synthetic folic acid consumed on an empty stomach (Gregory, 1997). Many folate-rich foods are fruits and vegetables, adequate intakes of which have been strongly associated with decreased risk for chronic disease (Steinmetz and Potter, 1996).

Inadequate calcium intake may result in weak bones, especially for women who have a greater risk of osteoporosis later in life. This point should be emphasized for young people who need to maximize their peak bone mass (Ueno et al., 2005), so an ideal time for prevention of osteoporosis may be during the college years. Female college students are suggested to be at high risk of insufficient calcium intake. They have lifestyles that are often characterized by eating out, irregular food intake patterns, skipping meals, and dieting (Wong and Huang, 1999).

In Croatian mountain region, milk and dairy products are traditionally present in a daily diet, which is not the case for the consumption of fruits and vegetables. The study with girls and boys at the age of 14-18 years examines milk and dairy products presence in boarding

school meals in Croatia. Mountain regions Lika and Gorski Kotar show the highest values of dairy products consumption (Gajdoš Kljusurić, 2003).
The aim of this study was to determine the average dietary folate and calcium intake among young people from mountain region, taking into account the importance of these two micronutrients in health.

SUBJECTS AND METHODS

Subjects

The study included a representative sample of people, gender and age considered, 321 university students both genders (50.2% females and 49.8% males), 19-25 years old, randomly selected. The subjects were students of Faculty of Teacher Education and University of Applied Sciences "Nikola Tesla" in Gospić, Lika-Senj County, Croatia. Participation was voluntary. Survey was in a classroom setting.

Food frequency questionnaires

To estimate the average folate intake a validated food frequency questionnaire (FFQ) was used (Colić Barić et al., 2009). The FFQ was designed to measure DFE. The DFE express all forms of dietary folates, including synthetic folic acid used in fortified foods and dietary supplements, as an amount that is equivalent to naturally occurring food folate. To calculate the DFE, the folate content of foods fortified with folate was multiplied by 1.7. The FFQ is a 39-item questionnaire that uses the previous month as a reference period with the following consumption frequencies: never, 1/month, 2-3/month, 1/week, 2-3/week, 4-6/week and every day. The subjects received the FFQ in the form of a booklet with incorporated food photographs (Senta et al., 2004). Each photograph showed small, medium, and large portion sizes. Folate intake was calculated using national food composition tables (Kaić-Rak and Antonić, 1990).
To estimate the average calcium intake a validated FFQ was used (Šatalić et al., 2007). The reference period for the FFQ was the previous year, and subjects were asked to recall how often they consumed certain foods. Options were never, 1/month, 2-3/month, 1/week, 2-3/week, 4-6/week, 1/day, and ≥ 2/day. The FFQ is a 27-item questionnaire. Portion size was defined using life-sized food photographs where for each food a small, medium, and large portion was shown (Hess, 1997), or portion size was described with a kitchen utensils. A Croatian food composition database was used for calculating calcium intake (Kaić-Rak and Antonić, 1990).

Results and Discussion

Subjects' mean age was 21.7 years. There was significant difference between women and men in mean age (22.1 vs. 21.3 years). Men had, as expected, a significantly higher mean BMI (25.0 kg/m^2) compared to women (22.1 kg/m^2) ($p<0.001$), and women had a significantly higher mean percent of body fat (27.8%) compared to men (15.0%) ($p<0.001$) (Table 1).

Table 1. Age and anthropometric characteristics of subjects (mean ± SD)

Parameters	Males	Females	Students	p
Number	160	161	321	
Age (years)	21.3 ± 2.2	22.1 ± 1.7	21.7 ± 2.0	<0.001 *
BMI (kg/m^2)	25.0 ± 6.2	22.1 ± 3.4	23.5 ± 5.2	<0.001 *
% of body fat	15.0 ± 5.7	27.8 ± 8.5	21.5 ± 9.7	<0.001 *

* Significant difference at $p<0.05$ according to t-test

The mean daily dietary folate intake was 239.8 ± 133.8 µg DFE, while the mean daily total folate intake, including folic acid from supplements, was 247.3 ± 144.4 µg DFE. The mean total folate intake was below recommendation (400 µg DFE) in both genders, 265.6 µg DFE

and 228.8 μg DFE in males and females, respectively. There was no significant difference in mean folate intake according to gender (p=0.205) (Table 2).

Table 2. Average daily folate intake according to gender (mean ± SD) (n=321)

Parameters	Males	Females	Students	p
Food folate (μg DFE)	261.4 ± 157.5	218.2 ± 100.6	239.8 ± 133.8	0.179
Food folate (% DRI)	65.3 ± 39.4	54.6 ± 25.1	60.0 ± 33.4	0.179
Supplements (μg DFE)	4.2 ± 37.8	10.6 ± 70.5	7.4 ± 56.5	0.482
Total folate (μg DFE)	265.6 ± 158.4	228.8 ± 126.6	247.3 ± 144.4	0.205
Total folate (% DRI)	66.4 ± 39.6	57.2 ± 31.7	61.8 ± 36.1	0.205

** Significant difference at p<0.05 according to t-test*

In a study with 100 women of childbearing-age (range 20–30 years) from Zagreb, Croatia, mean intake of naturally occurring food folate and folic acid from fortified cereals is 156.6 ± 72.2 μg/day assessed by 24-h recalls (Pucarin-Cvetković et al., 2006), which is lower than the intake found in this study. That survey proved that the values of folate and folic acid intake in a whole day meal were less than 180 μg, which does not meet the criteria for anaemia prevention and particularly not recommendations for prevention of neural tube defects in newborns. In a study of Šatalić et al. with students from all five Croatian University Centers (Zagreb, Split, Osijek, Rijeka and Zadar) mean folate intake is 442.9 μg i.e. 111% DRI in males and 373.4 μg i.e. 93% DRI in females, and it is significantly different according to gender (Šatalić et al., 2007). The mean folate intake in the Spanish students is 301.8 μg in males and 240.3 μg in females, and there is no significant difference between women and men in mean folate intake (Soriano et al., 2000). In a study of Van Diepen et al. participants also failed to consume adequate amounts of folate, 244.6 μg in Dutch and 263.0 μg in Greek university students (Van Diepen et al., 2011).

In this study the mean folate intake less than 100% DRI had 90.0% of females, and 81.3% had folate intake less than 75% DRI. The mean folate intake less than 100% DRI was determined in 85.7% of males, and 70.8% had intake less than 75% DRI (Figure 1). In a study with Croatian university students lower number of subjects have folate intake less than 100% DRI, 62% of females and 49% of males (Šatalić et al., 2007). Lewis et al. state that 68–87% of childbearing-age women population consume folate from foodstuffs in the amount less than 400 μg per day (Lewis et al., 1999).

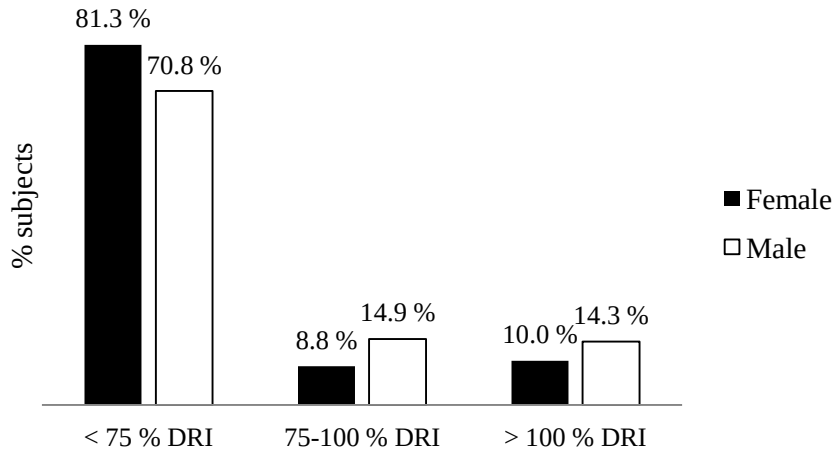

Figure 1. Average daily folate intake presented as a percentage of the DRI according to gender (% subjects)

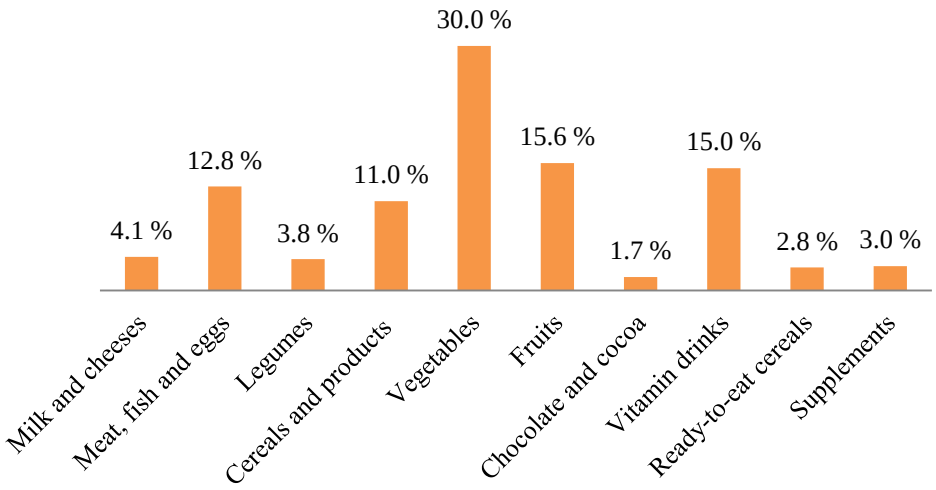

Figure 2. Food groups as main sources of folate in a daily diet

The main sources of folate in a daily diet were vegetables (30.0%), fruits (15.6%) and vitamin drinks (15.0%) (Figure 2). The highest correlation was determined between folate intake and intake of vegetables ($r=0.678$; $p<0.001$) and fruits ($r=0.572$; $p<0.001$). Fruit and vegetable intake was also favorably related to intake of dietary folate in Greek medical students (Bertsias et al., 2005).

The mean daily dietary calcium intake was 1062.0 ± 801.8 mg. No significant difference was determined for mean calcium intake between genders ($p=0.275$), 1110.8 mg (111.1% DRI) in males and 1012.9 mg (101.3% DRI) in females. The mean calcium intake was in accordance with recommendation in both genders (Table 3).

Table 3. Average daily calcium intake according to gender (mean ± SD) (n=321)

Parameters	Males	Females	Students	p
Food calcium (mg)	1110.8 ± 821.5	1012.9 ± 780.9	1062.0 ± 801.8	0.275
Food calcium (% DRI)	111.1 ± 82.2	101.3 ± 78.1	106.2 ± 80.2	0.275

** Significant difference at p<0.05 according to t-test*

The study with 161 healthy adults from the eastern part of Croatia shows that a mean calcium intake is 965 mg/day according to three FFQs, 909 mg for women and 1105 mg for men (Mandić-Puljek et al., 2005). There is a statistically significant difference between genders for daily calcium intake ($p=0.009$). Higher mean calcium intake, than in this study, is determined in a study with Croatian university students (1518.0 mg). Men have significantly higher mean calcium intake than women (1711.1 vs. 1444.4 mg) (Šatalić et al., 2007). Average calcium intake in a study with Spanish university students is 915.3 mg and 815.4 mg in males and females, respectively. There is no significant difference between women and men in mean calcium intake (Soriano et al., 2000), which is also determined in this study.

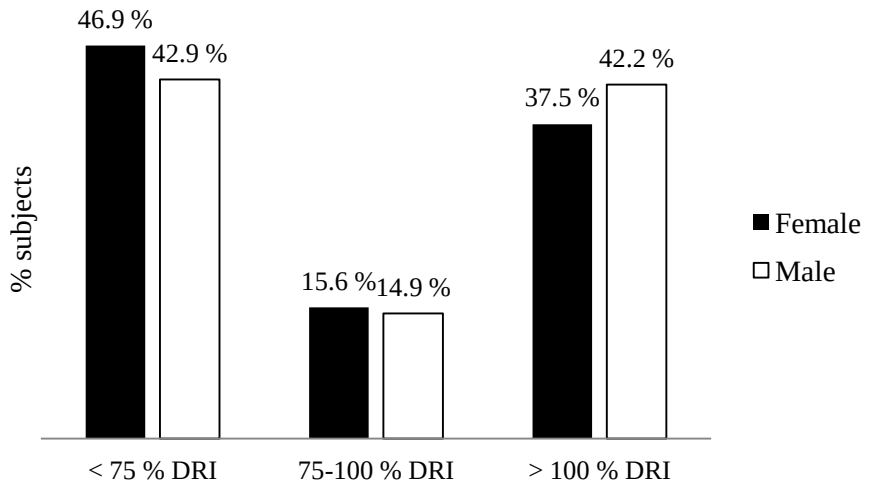

Figure 3. Average daily calcium intake presented as a percentage of the DRI according to gender (% subjects)

Mean calcium intake less than 100% DRI had 62.5% of females and 57.8% of males (Figure 3). Lower number of university students in a study of Šatalić et al. have calcium intake less than 100% DRI, 31% of females and 24% of males (Šatalić et al., 2007).

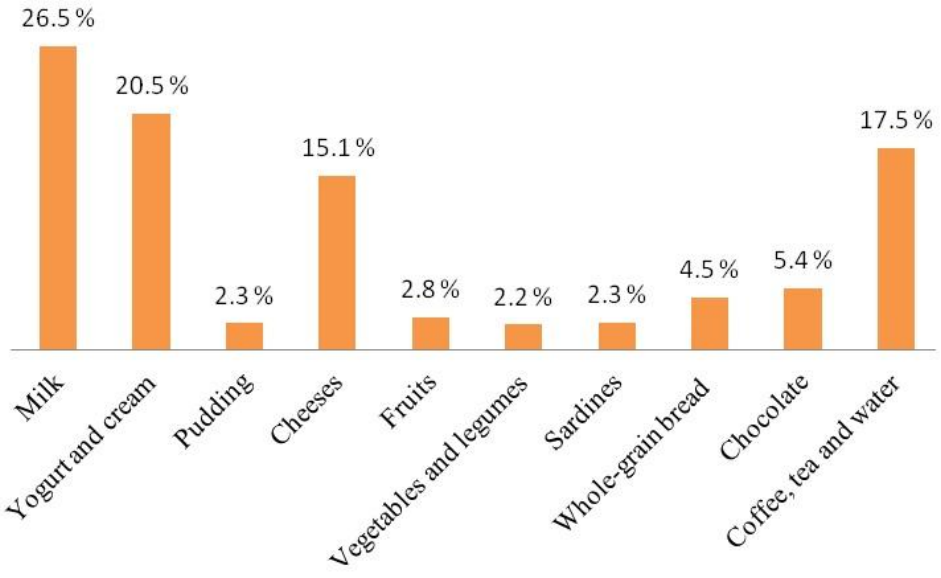

Figure 4. Food groups as main sources of calcium in a daily diet

Milk and dairy products (milk, yogurt, cream, cheeses and pudding) were the main source of calcium in a daily diet (64.4%) (Figure 4). Similar result is found in a study with healthy adults in eastern Croatia (61.3%) (Mandić-Puljek et al., 2005).

CONCLUSIONS

While milk and dairy products are traditionally present in their daily diet it was noticed that the average daily calcium intake was in accordance with the recommended value but the average daily folate intake was below recommended value because of the low consumption of fruits and vegetables. It is important to know the nutritional needs of population for assuring food security. Also, according the study the nutritional recommendations and some changes in daily dietary pattern are needed.

ACKNOWLEDGEMENT

This study is a part of Scientific Project “Diet, Homocysteine and Bone Health”, funded by the Ministry of Science, Education and Sport, Republic of Croatia.

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CONTENTS OF MAIN PHENOLICS AND ANTIOXIDATIVE CAPACITY IN FROZEN RASPBERRY FRUITS (*RUBUS IDAEUS* L.) FROM ARILJE GROWING AREA

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ABSTRACT: Individually quick frozen (IQF) fruits of the most abundant raspberry cultivars (Meeker and Willamette) in Western Serbia were randomly taken from the commercial cold storage in Arilje growing area, and analyzed for main phenolics and antioxidative capacity. All samples for freezing were harvested in the full ripening stage in the orchards in close proximity to the commercial cold storage, frozen by conventional freezing, and stored at -18°C, prior to use. Using high-performance liquid chromatography, high content of ellagic acid (from 13,99 to 27,01 mg/100 g fw for Meeker; from 26,15 to 35,22 mg/100 g fw for Willamette) and cyanidin (from 20,75 to 29,97 mg/100 g fw for Meeker; from 32,97 to 64,68 mg/100 g fw for Willamette) were determined. Significant amounts of pelargonidin (up to 7,08 mg/100 g fw), gallic acid (up to 4,42 mg/100 g fw), quercetin (up to 1,56 mg/100 g fw), and apigenin (up to 0,42 mg/100 g fw) were also detected in all samples. Although the raspberries cv. Willamette contain higher concentration of detected phenolics than raspberries cv. Meeker, nevertheless antioxidative capacity is nearly equal for both cultivars examined.
Key words: *commercial cold storage, Arilje growing area, Meeker, Willamette, IQF, anthocyanins, phenolics, antioxidative capacity, HPLC-DAD, ellagic acid, cyanidin*

INTRODUCTION

Red raspberries (*Rubus idaeus* L.) are soft, juicy fruits with a distinct aroma. It is an economically important berry crop that contains numerous bioactive compounds and natural antioxidants with a high free radical scavenging capacity. Berry fruits are extremely perishable and have a short market life. Therefore processing, in particular freezing, is necessary in order to provide raspberries to the market and consumers over the entire year.
In addition to vitamins and minerals, raspberries are also rich in anthocyanins, phenolic acids, and other flavonoids. Anthocyanins are the major contributors to the red colour pigment in berry fruits and are also used by consumers to judge the quality of a fruit. Mullen et al. (2002) have identified as many as 11 anthocyanins in raspberries. Flavonols (kaempferol, quercetin and myricetin) and phenolic acids (*p*-coumaric, gallic, ferulic and ellagic acids) have been also detected in berry fruits, with proposed beneficial effects on human health (Hertog et al., 1992; Horbowicz et al., 2008; Bobinaitė et al., 2012).
According to the International Raspberry Organization, the world's raspberry production in the period 2000-2010. is between 450.000 and 500.000 t (including Russia). In Serbia, the raspberries are being produced on 13.500 ha, and production is varying between 60.000 and 94.366 t. Arilje growing area is the main production center, with one quarter of the total raspberry production in Serbia (Leposavić et al., 2004).
The object of our study was to determine the chemical composition and antioxidative capacity in a commercial cold-storage frozen fruits of the most abundant raspberry cultivars (Meeker (5%) and Willamette (95%)) in Arilje growing area of Western Serbia (Petrović and Leposavić, 2011).

MATERIAL AND METHODS

Collecting samples

All samples for freezing were harvested in the full ripening stage in the orchards approximately 1 km away from the commercial cold storage, frozen the same day using the conventional freezing (classic) tunnel, and stored at -18°C (Petrović and Leposavić, 2011). Individually quick frozen (IQF) raspberry commercial packs of 2.5 kg were randomly taken from the cold storage from Arilje growing area of Western Serbia, as follows: three packs of cv. Meeker with harvesting dates: (*i*) 20.06.2010., (*ii*) 09.07.2010., (*iii*) 12.07.2010., and five packs of cv. Willamette with harvesting dates: (*i*) 24.06.2010., (*ii*) 14.07.2010., (*iii*) 17.07.2010., (*iv*) 19.07.2010., (*v*), 22.07.2010. Above-mentioned disproportion in number of packs is due to the different abundance of analyzed cultivars in Arilje growing area. Upon receipt at the Department for Fruit Processing Technology, the raspberries were stored at -18 °C until the analysis.

Soluble solid content (SSC), dry matter content (DM), sugar content, and titratable acidity (TA)

The SSC of the fruit was determined on a manual refractometer (3828, Carl Zeiss, Germany). The dry matter content was determined by drying at 105 ºC until constant mass. Titratable acidity (TA) was determined by neutralization of fruit extract with 0.1 N NaOH to pH 8.2, using phenolphthalein as indicator. Acidity was expressed as mg malic acid/100 g fresh weight. Sucrose, inverted sugars, and total sugars content were determined by Luff-Schoorl method (Tanner and Brunner, 1979).

Determination of anthocyanin content

The monomeric anthocyanin pigment content of the aqueous extracts was determined using the pH-differential method described previously (Torre and Barritt, 1977; Prior et al., 1998; Liu et al., 2002). Pigment content was calculated as micrograms of cyanidin-3-glucoside equivalents/100 g fresh weight (mg cyn-3-glu/100 g fw), using an extinction coefficient of 26.900 L/cm/mol and molecular weight of 449,2 g/mol.

Determination of flavonoid contents and total phenolics

Total flavonoid content was determined by a colorimetric method described previously (Zhishen et al., 1999; Liu et al., 2002). The results are expressed as micrograms of catechin equivalents/100 g fresh weight (mg CE/100 g fw).

The total phenolic content was determined using a modified Folin-Ciocalteu colorimetric method (Singleton et al., 1999; Liu et al., 2002), with results expressed as micrograms of gallic acid equivalents/100 g fresh weight (mg GAE/100 g fw).

Antioxidant activity

Antioxidant properties were determined by the ABTS and DPPH assays. $ABTS^{\bullet+}$ radical cation scavenging activity was determined according to the method described by Re et al. (1999). Antioxidant activity was determined using the DPPH method reported by Brand-Williams et al. with modifications (Sanchez-Moreno et al., 1998). Results were expressed as Trolox equivalent antioxidant capacity (mM TE/100 g fw).

Extraction and HPLC-DAD analysis

Samples were prepared according to the method of Hertog et al. (1992). Samples were analyzed using an Agilent 1260 series HPLC (Agilent Technologies, Santa Clara, CA, USA) linked to a ChemStation data handling system, using a ZORBAX Eclipse Plus C18 column (4.6 x 150 mm, 3.5 µm particles). Injection volume was 5 µL and the temperature was set at 30 ºC. Solvent A was 1% formic acid and solvent B was acetonitrile. The gradient used was as follows: 0–10 min, 10% of B in A; 10–25 min, 15–50% of B in A; 25–30 min, 50-80% of B in A; 30–32 min, 10% of B in A. By using this gradient (flow rate 0.5 ml/min), a good purity and separation was achieved in raspberry samples. The HPLC equipment was used with a

diode array detector (DAD). Ellagic acid (EA) and gallic acid (GA) were detected at 260 and 280 nm, respectively; apigenin and quercetin were detected at 329 and 360 nm, respectively; anthocyanins (cyanidin (CYA) and pelargonidin (PEL)) were detected at 520 nm. Phenolic compounds were identified according to peak retention time and UV/Vis spectra by comparing them with those of the standards. The quantities of the different phenolic compounds were based on peak areas, and expressed as mg/100 g fw.

Statistical analysis

For all the experiments, three samples were analyzed and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) to examine differences among the cultivars, using Statistica 7 (StatSoft, Inc., Tulsa, OK, USA). The pairwise comparisons between different parameters were performed out using Duncan's test ($p < 0.05$).

RESULTS AND DISCUSSION

Raspberry samples

Raspberries cvs. Meeker and Willamette were analyzed after four months of storage at -18°C. Given that the raspberry fruits ripen successively, the harvest is conducted on several occasions. The raspberry harvest season for the cvs. Meeker and Willamette lasts from 21 to 40 days, depending on the way of growing and agro-ecological condition of the raspberry growing area (Petrović and Leposavić, 2011). Taking this into account, randomly taken IQF raspberry packs of cvs. Meeker (from 20.06.2010. till 12.07.2010.) and Willamette (from 24.06.2010. till 22.07.2010.) from the commercial cold storage completely cover the entire harvesting periods.

Every single IQF raspberry pack was divided in three subsamples, in order to provide three replicate experiments. All results in this study are presented as mean value of all cultivar subsamples ± standard deviation (9 and 15 subsamples for Meeker and Willamette, respectively).

Chemical properties

Chemical properties of berry fruits contribute to fruit flavour. High sugars and high acids are required for good berry flavour.

Table 1. Chemical properties of frozen raspberries from commercial cold storage in Arilje growing area

	ANOVA	Meeker	Willamette
dry matter content - DM (%)	ns	14,17 ± 1,04 a	14,47 ± 0,95 a
soluble solid content - SSC (%)	ns	9,09 ± 1,26 a	9,95 ± 1,37 a
total sugars (%)	ns	5,54 ± 1,07 a	5,83 ± 0,90 a
inverted sugars (%)	ns	4,72 ± 0,96 a	4,92 ± 0,85 a
sucrose (%)	ns	0,79 ± 0,11 a	0,86 ± 0,09 a
titratable acidity (%)	***	1,73 ± 0,21 b	2,15 ± 0,13 a
pH	**	2,86 ± 0,06 a	2,78 ± 0,06 b
sugar/acid ratio	ns	3,30 ± 0,97 a	2,71 ± 0,41 a

Values with a different letters denote statistically significant differences (Duncan's test, $p < 0.05$).
ns,,**,***: non significant or significant at $p < 0.05, 0.01, 0.001$, respectively.*

For instance, high acid and low sugar level result in a tart berry, while high sugar and low acid level result in a bland taste. Moreover, low levels of acid and sugar result in tasteless berries (Wang et al., 2009). Chemical properties of Meeker and Willamette commercially available frozen samples are presented in Table 1. As can be noticed, there are no statistically significant differences between the cultivars examined. As for the titratable

acidity, cv. Willamette has a higher total acids content compared to cv. Meeker, that is already reported (Finn and Lawrence, 2001).
In comparison, the results of chemical properties in our study are in excellent agreement with reports by other researchers (Finn and Lawrence, 2001; Stanisavljević et al., 2002; Kafkas et al., 2008).

Contents of bioactive compounds and antioxidative capacity
The total anthocyanins, total flavonoids, and total phenolics were measured for all the samples. The results are given in Table 2.
The total anthocyanin and total flavonoid contents showed great variation in different raspberry cultivars (Willamette had higher anthocyanin and flavonoid contents, followed by Meeker). On the other hand, regarding the total phenolic content there is no statistically significant difference between the cultivars examined. These results are in great agreement with results by other researchers (Anttonen and Karjalainen, 2005; Wang et al., 2009; Sariburun et al., 2010). Anttonen and Karjalainen (2005) reported that the total anthocyanin and total phenolic contents ranged from 0 to 51 mg/100 g fw and from 192 to 359 mg/100 g fw, respectively, for seventeen raspberry cultivars grown at two different farms in Finland. Bobinaitė et al. (2012) found in raspberry cv. Meeker that the total anthocyanins and total phenolics were 44,3 and 388,8 mg/100 g fw, respectively. However, it is known that the contents of bioactive compounds in fruits and vegetables depend on various factors, such as genotypic differences, pre-harvest climactic conditions and post-harvest handling procedures.

Table 2. Total anthocyanin, total flavonoid and total phenolic contents (mg/100 g fw), and free radical scavenging parameters (mM TE/100 g fw) of frozen raspberries from commercial cold storage in Arilje growing area

	ANOVA	Meeker	Willamette
total anthocyanins	***	44,04 ± 4,05 b	87,04 ± 10,09 a
total flavonoids	***	43,17 ± 4,82 b	69,41 ± 9,78 a
total phenolics	ns	358,77 ± 39,81 a	346,99 ± 42,37 a
ABTS	ns	278,71 ± 18,96 a	255,93 ± 37,08 a
DPPH	ns	27,15 ± 1,83 a	27,34 ± 1,54 a

Values with a different letters denote statistically significant differences (Duncan's test, $p < 0.05$). ns,,**,***: non significant or significant at $p < 0.05$, 0.01, 0.001, respectively.*

The results for the antioxidant capacity (ABTS and DPPH assays) are also given in Table 2. As can be noticed, no statistically significant differences were found amongst these two raspberry cultivars. Since there are a large number of different types of antioxidant compounds that might contribute to the total antioxidant capacity, it is not clear which components are responsible for the observed antioxidative capacity. The antioxidant capacity of raspberry fruits appears to be largely influenced by the polyphenolics, rather than anthocyanins and flavonoids. These data are in agreement with other reports in the literature (Sariburun et al., 2010; Novaković et al., 2011).
Analyzing HPLC-DAD chromatograms of aqueous methanol extracts of frozen raspberries cvs. Meeker and Willamette, six peaks were clearly identified. Some of the peaks are presented in Figure 1. Identified compounds were quantified and summarized in Table 3.

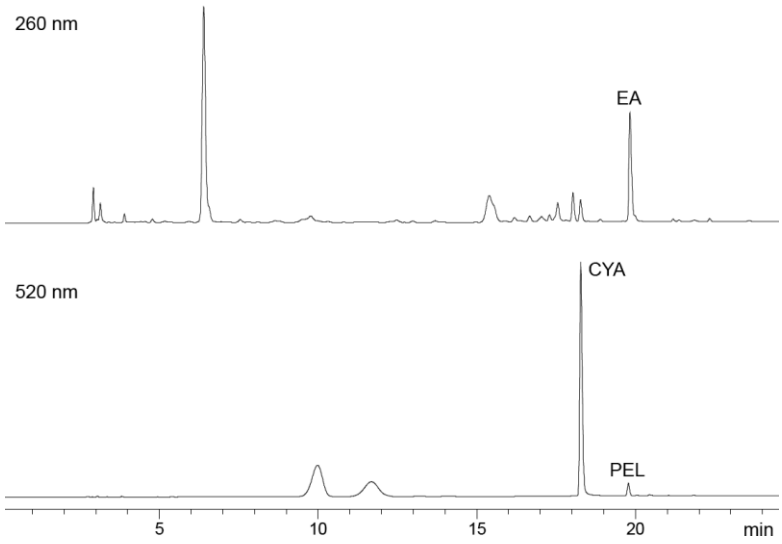

Figure 1. HPLC chromatogram for the quantification of ellagic acid (EA) monitored at 260 nm (top), and cyanidin (CYA) and pelargonidin (PEL) monitored at 520 nm (bottom)

It was found that the amount of quercetin in Meeker is five times lower than that in Willamette. Surprisingly, certain amount of apigenin is found in both cultivars. Kaempferol and myricetin were not detected in any of the samples. Relatively high content of free ellagic acid was found (19,59 and 28,90 mg/100 g fw for Meeker and Willamette, respectively). These results are comparable with the findings previously reported (Anttonen and Karjalainen, 2005; Bobinaitė et al., 2012). The main anthocyanin found in raspberry is cyanidin (23,99 and 53,42 mg/100 g fw for Meeker and Willamette, respectively), followed by pelargonidin. Comparable results are reported in the literature (Horbowicz et al., 2008; Wang et al., 2009).

Table 3. Contents of main phenolics (mg/100 g fw) in frozen raspberries from commercial cold storage in Arilje growing area

	ANOVA	Meeker	Willamette
apigenin	***	0,23 ± 0,06 b	0,36 ± 0,05 a
quercetin	***	0,18 ± 0,03 b	0,96 ± 0,37 a
gallic acid	***	2,73 ± 0,21 b	3,68 ± 0,55 a
ellagic acid	***	19,59 ± 5,07 b	28,90 ± 2,77 a
cyanidin	***	23,99 ± 4,10 b	53,42 ± 11,32 a
pelargonidin	***	2,47 ± 0,60 b	5,16 ± 1,42 a

Values with a different letters denote statistically significant differences (Duncan's test, $p < 0.05$). ns,,**,***: non significant or significant at $p < 0.05, 0.01, 0.001$, respectively.*

CONCLUSIONS

In conclusion, commercially available frozen raspberries cvs. Meeker and Willamette were randomly taken from the commercial cold storage in Arilje growing area of Western Serbia, and analyzed by high performance liquid chromatography. Numerous bioactive compounds, such as apigenin, quercetin, ellagic acid, gallic acid, cyanidin, and pelargonidin were identified and quantified, in both cultivars examined. Furthermore, free radical scavenging capacity assays were performed and it was found that antioxidative capacity is nearly equal for both cultivars examined, although the raspberries cvs. Meeker and Willamette contain different concentrations of detected phenolics.

ACKNOWLEDGEMENTS

This work was financially supported by the Ministry of Agriculture, Trade, Forestry and Water Management (STAR Project-AAP057) and Ministry of Education and Science of the Republic of Serbia (Project TR-31093).
The authors thank Mirjana Pajić for chemical analysis.

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OPTIMISATION AND APPLICATION OF ANTIHEMOLYTIC ASSAY ON *FAGOPYRI HERBA* EXTRACTS

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ABSTRACT: *Fagopyri herba*, a herbal drug derived from common buckwheat (*Fagopyrum esculentum* Moench) is a very rich source of various phenolic compounds which are known as potential antioxidants. The main active compound is a flavonol glycoside rutin. The aim of this study was to investigate antihemolytic activity of the *Fagopyri herba* extracts as a parameter which indicates their antioxidant potential in biological systems. Antihemolytic activity assay measures the ability of a tested extract to inhibit hemolysis of erythrocytes in buffer solution caused by oxidative damage of their cell membrane. Since this test is highly dependent on various parameters (cell suspension concentration, species from which the blood was taken, concentration of reagents, type of extract), they were optimized for testing the investigated water and ethanol *Fagopyri herba* extracts. Optimal concentration of hydrogen peroxide solution was found to be 0.0625% (v/v) while 2 μM of sodium azide was added to the erythrocyte suspension to inhibit catalase enzyme. Ethanolic extract showed lower IC_{50} value (3.46±0.185 mg/mL) in comparison to water extract (42.2±7.27 mg/mL), which is in accordance with their rutin content determined by high performance liquid chromatography.
Key words: *Fagopyri herba, antihemolytic assay, method optimisation*

INTRODUCTION

The pathology of numerous chronic diseases involves oxidative damage of cellular components. (Ebrahimzadeh et al., 2010). Carcinogenesis, coronary heart disease, and many other health problems are related to activity of reactive oxygen species (ROS) which affect lipids in cell membranes, proteins in tissues, enzymes, carbohydrates and DNA. Antioxidants prevent tissue damage by preventing the formation of free radicals, scavenging them, or by promoting their decomposition. Antioxidant system in humans includes antioxidants produced in the body (endogenous) and antioxidants obtained from the diet (exogenous) (Pier-Giorgio, P., 2000). The most important exogenous antioxidants are phenolic compounds from different grains, fruit and vegetables.

Common buckwheat (*Fagopyrum esculentum* Moench) is important part of human diet. This pseudocereal is known as a very rich source of antioxidants. Since buckwheat seeds have been extensively investigated (Oomah et al., 1996; Watanabe et al., 1997; Watanabe et al., 1998; Sedej et al., 2010; Sedej et al., 2011) it was interesting to examine its herbal drug as a source of valuable phenolic compounds. Recent studies reported rutin as the main antioxidant compound in *Fagopyri herba* (Baumgertel, et al., 2010).

Over the last decades, numerous tests were developed for determination of antioxidant capacity in various test systems (Miller H. E., 1971; Brand-Williams et al., 2005; Magalhaes et al, 2008), but many of these tests performed in *in vitro* conditions fail to properly assess the biological activity of the investigated compound. Antihemolytic activity assay measures the ability of a tested extract to inhibit hemolysis of erythrocytes in buffer solution caused by oxidative damage of their cell membrane, and therefore, may indicate the potential biological activity of the investigated compound or extract.

Since this test is highly dependent on various parameters (cell suspension concentration, species from which the blood was taken, concentration of reagents, type of extract), the aim

of this work was to optimize these parameters for testing the investigated water and ethanol *Fagopyri herba* extracts.

MATERIAL AND METHODS

Materials

Herbal drug (*Fagopyri herba)* was obtained from the Institute for Medicinal Plants Research "Dr Josif Pančić" from Belgrade.
Crude plant extracts were obtained by maceration (24 h, room temperature) with water and ethanol/water mixture (50:50, v/v), with the ratio of raw materials to solvent of 1:25.
Blood samples were obtained from healthy volunteers by venipuncture, after obtaining informed consent.

Reagents and chemicals

Phosphated buffer solution (PBS, pH=7.4) was made by dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in deionised water to a total volume of 1L in a volumetric flask. Hydrogen-peroxide solution was made by diluting 2.5 ml of 30% H_2O_2 with PBS solution to a total volume of 50 ml in a volumetric flask and subsequently diluting the obtained solution eight times to a final concentration of 0.19% (which corresponds to 0.063%, or 32.5 µM, of H_2O_2 when added to reaction mixture). Sodium-azide solution (0.002 M) was made by dissolving 0.013 g of sodium-azide in PBS solution to a total volume of 100 ml in a volumetric flask.
Methanol (HPLC grade) and formic acid were supplied by Merck KGaA (Darmstadt, Germany). Water used for HPLC analysis was purified using a Millipore, Elix UV and Simplicity Water Purification System (Milford, MA, USA).

Antihemolytic assay-optimized method

Antihemolytic activity of the extracts was determined using a modification of method by *Ko et al* (1997). Blood (4-6 ml) was drawn into heparinated vacuum tubes and centrifuged at 1000×g for 10 minutes, after which the plasma and buffy coat were removed. Remaining erythrocytes were washed three times with PBS and centrifuged to remove the buffer. The remaining cell cake was gently resuspended in sodium-azide solution in PBS (to achieve inhibition of catalase enzyme) to obtain approximately 10% hematocryte. 0.5 ml of the erythrocyte suspension was added to the test tube, after which 0.5 ml of the buckwheat extract dissolved in PBS was added, and the same procedure was followed for all examined concentrations of each extract. Hydrogen-peroxide solution (0.5 ml; 0.0625%) was added to the test tubes, after which they were gently shaken and incubated in water bath at 37^oC during two hours, being periodically gently shaken by hand to facilitate the hemolysis process. For every investigated extract, a "zero hemolysis" sample was also prepared by adding the erythrocyte suspension and PBS extract solution to a test tube, without the addition of hydrogen-peroxide (0.5 ml of PBS solution was added instead). Also, another sample ("uninhibited hemolysis") was prepared by adding hydrogen-peroxide solution to erythrocyte suspension, without adding the investigated extract (0.5 ml PBS solution was added instead of extract). After incubation period, 200 µl of every sample was transferred to an empty test tube and diluted with 3.8 ml of PBS solution. After centrifuging at 1000×g for 10 minutes to eliminate the unhemolyzed erythrocytes, absorbance of the supernatant (marked as A) was measured at 540 nm using a spectrophotometer. Also, 200 µl of every sample was diluted with 3.8 ml of cold distilled water to achieve a total hemolysis, and after centrifuging the absorbance of the supernatant was also measured spectrophotometrically at 540 nm (marked as B). The extent of the hemolysis in every sample was calculated as:

A/B×100 = % of hemolysis

Since the percentage of hemolysis was calculated for all sample concentrations, these values were plotted against sample concentrations and, using linear regression, IC_{50} values of every investigated extract were calculated.

HPLC analysis

Rutin content of the examined extracts was determined according to method reported by Mišan et al. (2011) using a liquid chromatograph (Agilent 1200 series), equipped with Eclipse XDB-C18, 1.8 µm, 4.6×50 mm column (Agilent).

RESULTS AND DISCUSSION

In order to optimize the antihemolytic test conditions, two concentrations of hydrogen-peroxyde of 0.375% and 0.190% (corresponding to final concentrations of 0.125% and 0.063% in the reaction suspension, respectively) were selected for testing. The initial tests showed excessive foam formation after addition of hydrogen peroxyde to the erythrocyte suspension due to rapid scavenging of hydrogen peroxyde by catalase enzyme, which proved problematic and could lead to inconsistent analytical results. To overcome this effect, erythrocyte suspension was subsequently made with PBS solution in which 0.002 mol×dm^{-3} of sodium azide was added to inhibit the catalase enzyme. Concentration of hydrogen peroxyde of 0.375% caused very rapid onset of hemolysis (95.5% hemolysis after first hour) which was unsuitable for further testing. The addition of lower peroxyde concentration of 0.19% to the reaction suspension yielded favorable results of hemolysis (62.7% after 1h, 84.4% after 2h and 89.9% after 3h of incubation) in hemolysis conditions without added antioxidants. Therefore, this concentration of hydrogen peroxyde was chosen for further tests.

Optimal incubation time was determined using water extract of *Fagopyri herba* in the above chosen hemolysis system. Its antihemolytic activity was monitored during three hour period (Fig 1). As it can be seen from the obtained results, *Fagopyri herba* water extracts inhibited the hemolysis of erythrocytes in the presence of 0.063% hydrogen-peroxyde in a concentration dependant manner. Incubation time of two hours was chosen as optimal since, by that time, the suspension not containing hydrogen peroxyde reached more than 80% hemolysis.

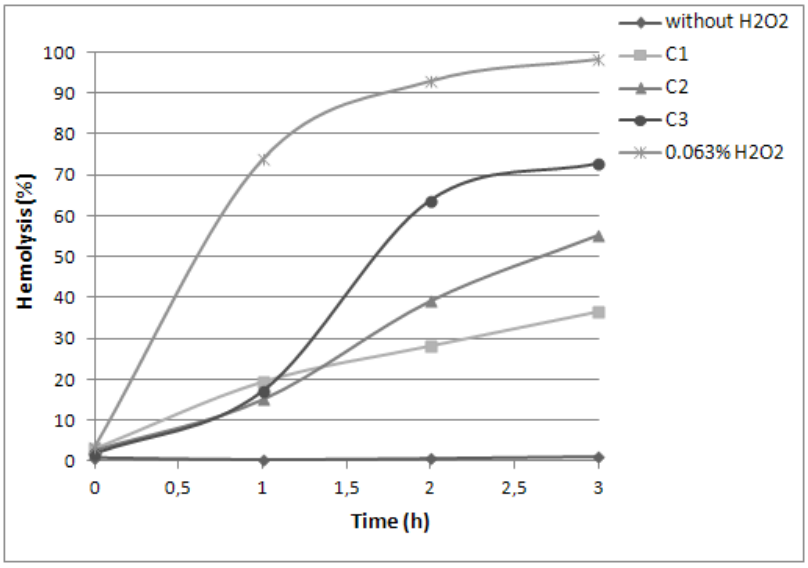

Fig 1. Antihemolytic activity of three different concentrations of *Fagopyri herba* water extract

After optimization of all necessary parameters, optimized antihemolytic assay was performed on water and ethanol/water *Fagopyri herba* extracts in order to test its applicability on different extract types.

The effectiveness of the tested extracts regarding antihemolytic activity could be expressed by IC_{50} value which is defined as a concentration of the investigated extract at which 50% of hemolysis inhibition is achieved. The obtained value of hemolysis (%) was corrected for the

hemolysis of the control suspensions (without added extract and without added hydrogen peroxyde) and that values were plotted against the concentration of the extract. Linear regression analysis was used to calculate IC_{50} values (Table 1).

Table 1. Concentration range, linear regression curves, IC_{50} values and rutin content of water and ethanol/water (50/50 v/v) *Fagopyri herba* extracts

Type of extract	Concentration range (mg/mL)	Linear regression curve; R^2	IC_{50} (mg/mL)	Rutin content (mg/g sample)
water	1.34-4.02	y = -1.2027x+96.82; R^2=0.9966	42.2±7.27	0.251
ethanol/water (50/50 v/v)	1.35-4.04	y = -10.734x + 88.082; R^2=0.9967	3.46±0.185	40.35

Lower IC_{50} values indicate higher antihemolytic activity of ethanol/water extracts which is in accordance with its considerably higher content of rutin.

CONCLUSIONS

The obtained results show that the optimal concentration of hydrogen peroxyde was found to be 0.063%, which causes between 80% and 90% of hemolysis of erythrocytes in the test suspension after two hours of incubation at 37°C. Addition of 0.002 mol×dm^{-3} of sodium azide to erythrocyte suspension was found to cause an efficient inhibition of the catalaze enzyme in order to prevent rapid decomposition of the added peroxyde. The investigated *Fagopyri herba* water and ethanolic extracts showed concentration dependent inhibition of hemolysis in the tested system, with IC_{50} values of 42.2±7.27 mg/mL and 3.46±0.185 mg/mL, respectively. These results were in accordance with the rutin content of these extracts determined by high performance liquid chromatography. It can be concluded that the antihemolytic activity test can be successfully applied to analysis of *Fagopyri herba* extracts, which showed very good antihemolytic activity in such test system, and this can be further indication of the beneficial biological activity of these extracts.

ACKNOWLEDGEMENTS

This work is a part of the National Project (TR–31029) financially supported by the Ministry of Education and Science, Republic of Serbia.

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POTENTIAL ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF MULBERRY FRUITS (*MORUS ALBA* L.) GROWN IN SERBIA

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ABSTRACT: Mulberry trees are distributed throughout Serbia. Besides their use in forage and food for animals, they are also used as herbal medicines. This study is carried out to determine total phenolics content, total flavonoids content, total monomeric anthocyanins, as well as DPPH scavenging activity, reducing power and minimum inhibitory concentration (MIC) of the fruits of mulberry species (*Morus alba* L.) grown in Serbia. Fresh fruits of mulberry were extracted by four different solvents (water, ethanol, methanol and acetone). Determination of fruit weight, total soluble solids, total dry weight, pH and acidity was performed. The total phenolics content (TPC) and total flavonoids content (TFC) in mulberry extract were determined spectrophotometrically. The total monomeric anthocyanins (TMA) were estimated by a pH differential method. Antioxidant activity (AA) of mulberry extracts was evaluated using DPPH* radical scavenging method. The reducing power of extracts and standard (ascorbic acid) were determinate by Oyaizu method. MIC of the extract and cirsimarin against tested bacteria were determined based on a microdilution method. Methanolic mulberry fruits extract with the highest amount of total phenolics component was the most potent antioxidant in all the assays used. On the other hand, MIC determination showed that all other extracts have higher antimicrobial activity. This is the first report of *Morus alba fructus* grown in Serbia and determination of the potential antioxidant and antimicrobial activity of mulberry fruits extract. This information will be of considerable value to the commercial producers of mulberry trees, cultivation or pharmaceutical industry for potential new mulberry supplement production.
Key words: *mulberry fruits, phenols, flavonoids, antioxidant activity, antimicrobial activity*

INTRODUCTION

Plants have received a lot of attention as sources of biologically active substances including antioxidants, antimutagens and anticarcinogens (Diliard et. al., 2000). However, scientific information on antioxidant properties of various plants, particularly those that are less widely used in culinary and medicine are still limited. Therefore, the assessment of such properties remains an interesting and useful task, particularly for finding new sources for natural antioxidants, functional foods and nutraceuticals (Miliauskas et al., 2004; Arabashahi-Delouee et al., 2006).

Mulberry (*Morus alba* L) plant was originally grown in China and widely has been used over thousands of years in folk medicine for its multiple medicinal properties. All species of *Morus* play role both in agriculture and in medicine (Butt et al., 2008). Recently, it has also started to gain an important position in the food industry due to the increasing findings of its healthy benefits such as reduced risk of certain types of cancer, coronary heart disease, stroke, high blood glucose level and ageing (Doi et al., 2000; Harauma et al., 2007; Jia et al., 1999; Lin and Lai, 2009). *M. alba* fruit is a good source of several phytonutritients and contains high amounts of phenols, flavonoids, and ascorbic acid (Zhishen et al., 1999; Tapiero et al., 2002; Bae and Suh, 2007, Memon et al., 2010).

The purpose of the present study was to investigate the effects of used solvent on the antioxidant and antimicrobial capacity and levels of extracted antioxidant compounds in extracts of white mulberry fruits from Serbia. This information will be of considerable value to the commercial producers of mulberry trees in the country.

MATERIAL AND METHODS

Chemicals and reagents

1,1-Diphenyl-2-picryl-hydrazyl-hydrate (DPPH), Folin–Ciocalteu reagent were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Chlorogenic acid and rutine were purchased from Sigma (Sigma, St. Luis, MO, USA). Aluminium chloride hexahydrate, anhydrous sodium carbonate, and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical reagent grade.

Sample preparation

Mulberry fruits were harvested from selected *M. alba* from Novi Sad, Serbia. Voucher specimens (*Morus alba* L. No 2-1794, Kać, UTM 34TDR211 det.: Goran Anačkov) were confirmed and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad, Serbia (Holmgren and Holmgren, 2003). The fruits were then stored in polyethylene bags at -20°C (up to 1 month) until analysis. Samples were ground in the blender before the extraction.

Fruit slurries were extracted with water and diferent buffers containing acetone/ethanol/methanol, water and acetic acid (70:29.5:0.5, v/v/v). Friut-solvent extraction ratio was 1:10 (w/v). The extraction process was carried out using ultrasonic bath (B-220, Brason and Smith-Kline Company, USA) at the room temperature for 1 hour. After filtration solvent was removed by rotary evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia) under vacuum, and was dried at 60°C to the constant mass. Dry extracts were stored in the glass bottles at 4°C to prevent oxidative damage until analysis.

Determination of fruit weight, moisture, total dry weight, total soluble solids, pH and acidity (of mulberry fruits)

All analyses were performed on 20 samples. Fruit weight was measured by using a digital balance with a sensitivity of 0.0001 g (Kern, Germany). The moisture of samples was determined by drying at 105 ± 2°C until they reached constant weight (AOAC, 1984). Total dry matter of the fruits was determined according to the methods of AOAC (1984). Total soluble solid contents (TSS) were determined by extracting and mixing one drop of juice from each fruit into a digital refractometer (Model ATR-ST, Schmidt-haensch, Germany) at 23.9°C. The pH measurements were made using a digital pH meter (Crison, Basic 20+, Espanola) calibrated with pH 4 and 7 buffers. Titratable acidity (TAc) was measured by the titrimetric method (AOAC, 1984). Titratable acidity of mulberry was expressed as % citric acid. TSS/TAc results are also determined.

Determination of antioxidant compounds in mulberry extracts

The total phenolics content (TPC) in the extracts was determined by the Folin–Ciocalteu method (Singleon, 1965; Kahkoen et al., 1999) and was expressed as mass (mg) of chlorogenic acid equivalents (ECA) per mass (g) of dry extracts. Triplicate tests were conducted for each sample. The total flavonoids (TFC) content has been determined by aluminium chloride colorimetric assay (Ercisli, Orhan, 2007), using rutine as a standard. It has been expressed as mass (mg) of rutine equivalents (ER) per mass (g) of dry extracts. Triplicate tests were done for each sample. Total monometric anthocyanins (TMA) were estimated by a pH differential method (Giusti and Wrolstad, 2005) using a VIS spectrophotometer (Janwey 6300, Germany). Absorbance was measured at 533 and 700 nm in buffers at pH 1.0 and 4.5 using molar extinction coefficient of 29,600. Results were expressed as mg of cyanidin-3-glucoside equivalent in g of dry extract (mg cy-3-glu/g). The results are presented as the mean value of three measurements.

DPPH assay and determination of reducing power of mulberry extracts

The free radical scavenging activity of mulberry extracts was determined as described by Espin (2000). This activity was also expressed as the inhibition concentration at 50% (IC_{50}), the concentration of test solution required to give 50% of decrease in absorbance compared

to the blank sample. The reducing power of mulberry extracts and ascorbic acid were determined by Oyaizu method (1986). Absorbances of samples were measured at 700 nm. Higher absorbance indicates a higher reducing power (reducing capability).

Statistical Analysis

Statistical analysis was carried out using Statistica 6.0. (StatSoft Inc, Tulsa, OK, US). All experiments were performed at least in triplicate unless specified otherwise. Results are presented as mean value ± standard deviation (SD). Significant levels were defined at $p<0.05$.

Microdilution method, determination of minimum inhibitory concentration (MIC) of extracts

The minimal inhibitory concentration (MIC) of the extract and cirsimarin against tested bacteria were determined based on a microdilution method in 96 multi-well microtiter plates **(**Satyajit et al., 2007) Standard antibiotic Amracin was used to control the sensitivity of the tested bacteria, where as Ketokonazol was used as control against the tested yeast. Color change was then assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated and that was the MIC for the tested samples.

RESULTS AND DISCUSSION

The fruit weight, fruit moisture, total dry weight (TDW), total soluble solids (TSS), pH and total acidity (TAc) of mulberry fruits are given in Table 1. Monitored parameters indicate that white mulberry has favorable characteristics for the experimental tests. All measured parameters were within the limits of other studies done on mulberry fruit (Ercisli and Orhan, 2007; Butt et al., 2008).

Table 1. Fruit weight, moisture, TDW, TSS, pH and TAc of mulberry fruits

Spacie	Frut weight [g]	Moisture [%]	pH	TDW [%]	TSS [%]	TAc [%]
Morus alba	2.4543±0.2612	75.18±1.00	5.99±0.02	24.82±1.00	17.85±0.07	0.16

As a chemical structure of phenolic compounds is most responsible for their antioxidant activity, measurement of total phenolics content could be related to antioxidant properties of investigated material. Total phenolics content (TPC), total flavonoids content (TFC), total flavonoids/total phenolics ratio (TFC/TPC) and contant of total monometric anthocyanins (TMA) are presented in the Table 2. Antioxidant potential (DPPH assay and reducing power of mulberry extract) are presented in the same table, also.

Table 2. Total phenols content (TPC), total flavonoids content (TFC), ratio TFC/TPC, total monometric anthocyanins (TMA) and antioxidant activity of mulberry extracts

Solvent	TPC [mg ECA/g]	TFC [mg ER/g]	—	[%]	TMA [mg cy-3-glu/g]	IC_{50} mg/ml	EC_{50} mg/ml
Methanol	8.61±0.19	3.42±0.01	39.72		0.78±0.01	0.2037	0.9615
Ethanol	8.27±0.15	3.53±0.01	42.68		0.53±0.01	0.2465	0.9397
Acetone	8.30±0.19	3.60±0.02	43.37		0.81±0.02	0.2783	0.9713
Water	8.06±0.17	3.86±0.06	47.89		0.35±0.02	0.4281	1.7750

Similar content of total phenols was observed in all extract, around 8 mg ECA/g. The highest content of flavonoids was detected in water extract, 3.86 mg ER/g. The ratio TFC/TPC in

wather extract was some higher than that in the other three extracts. The highest content of total monometric anthocyanins was detected in acetone extract, 0.81 mg cy-3-glu/g. Phenolics, flavonoids and anthocyanins can play a double role in reducing the rate of oxidation, as they participate in iron chelation and trapping radicals (Van Acker et al., 1998). Scavenging effect of mulberry extracts on DPPH˙ radicals increased with the increase of concentrations. Radical scavenging activity was found to exhibit 50% of inhibition value (IC_{50} value) at the extract concentration of 0.4281 mg/ml for investigated water extract (Table 2). IC_{50} values for other extracts were lower than 0.3 mg/ml. As IC_{50} values of all investigated samples could be considered as low, this implicates that all dried extracts could be recommended as antioxidant products. Ferrous ion, which commonly exists in food systems, is well known as an effective pro-oxidant component (Fraga and Oteiza, 2002). For the measurements of the reductive ability $Fe^{3+} \rightarrow Fe^{2+}$ transformation, in the presence of extracts have been investigated. Reducing power of investigated extracts were compared to reducing power of standard antioxidant compounds, Trolox and vitamin C. It is clear that both, Trolox and vitamin C, have better reductive capabilities than investigated extracts. EC_{50} value for vitamin C and Trolox was 0.046 mg/ml and 0.079 mg/ml, respectively. These values are much lower than EC_{50} values of investigated extracts (Table 2). The lowest value EC_{50} has been determined for ethanol extract, 0.9397 mg/ml, but it is still much higher concentration in comparison to EC_{50} values of standard antioxidant compounds vitamin C and Trolox. In comparison to ethanol extract, EC_{50} of other extracts were higher, indicating lower reducing capabilities.

The results on antimicrobial activity obtained by the dilution method are given in Table 3. Minimum inhibitory concentration (MIC) was determined for 15 indicator strains. The results suggest that the all extract of mulberry fruits showed antimicrobial activity within the concentration range of 15.62 to 125 µg/ml. The highest susceptibility to the water extracts was exhibited by *Klebsiella pneumonia* and *Pseudomonas glycinea*, (MIC=15.62 µg/ml), among the test bacteria, and by *Aspergillus glaucus* in mulberry extracts prepared with ethanol and acetone (MIC=15.62 µg/ml), among the test fungi. Conversely, the lowest susceptibility was observed in *Bacillus mycoides* in ethanolic extract (MIC=125 µg/ml) and in the fungi *Trichoderma viride* and *Phialophora fastigiata*, the measured MIC value being 125 µg/ml.

Table 3. Minimum inhibitory concentration (MIC) of mulberry extracts

Microorganism	MIC (µg/mL)					
	Mulberry sample				Standard	
	Ethanol	Methanol	Acetone	Water	Amracin	Ketokonazol
Staphylococcus aureus ATCC 25923	62.5	125	62.5	31.25	0.98	/
Echerichia coli ATCC 25922	31.25	62.5	31.25	31.25	0.49	/
Micrococcus lysodeikticu ATCC 4698	62.5	62.5	62.5	31.25	0.98	/
Bacillus subtilis FSB 2	125	125	125	62.5	1.95	/
Bacillus mycoides FSB 1	62.5	62.5	62.5	62.5	1.95	/
Klebsiella pneumoniae FSB 26	31.25	62.5	31.25	15.62	0.24	/
Pseudomonas glycinea FSB 40	62.5	62.5	31.25	15.62	0.98	/
Candida albicans ATCC 10259	125	62.5	62.5	62.5	/	0.98
Penicillium canescens FSB 24	125	62.5	62.5	62.5	/	0.98
Aspergillus niger FSB 31	15.62	62.5	15.62	31.25	/	0.24
Fusarium oxysporum FSB 91	62.5	62.5	62.5	62.25	/	1.95
Aspergillus glaucus FSB 32	31.25	62.5	31.25	62.5	/	0.49
Alternaria alternata FSB 51	62.5	62.5	62.5	62.5	/	1.95
Trichoderma viride FSB 11	125	62.5	62.5	62.5	/	1.95
Phialophora fastigiata FSB 81	125	125	125	125	/	1.95

CONCLUSION

The results of this study clearly indicated that methanol, ethanol, acetone and water mulberry extracts were good scavengers of synthetic DPPH radicals what indicates that they all could be used as antioxidant products. Also, they all possess reductive capabilities. They all are adequate source of phenolic and flavoniods compounds, compounds well known as an antioxidants with high antioxidant activity. The obtained results suggest that the extracts of the species *Morus alba* L. possess antimicrobial activity under *in vitro* conditions against the test fungi and bacteria. Our study has shown that mulberry extracts prepared with different solvents had similar characteristics . We recommend ethanol and water to be used as solvents, because these solvents are not toxic to human body. Antioxidant and antimicrobial properties of various extracts of many plants are of great interest in both fundamental science and food industry, since their potential use as natural additives has emerged from a growing tendency to replace synthetic antioxidants by natural ones. The present study confirmed the antimicrobial and antioxidant activities of the extracts of the Serbian plant *Morus alba* L.

ACKNOWLEDGEMENTS

Financial support of this work by the Serbian Ministry of Science, Project No. TR 31013, is gratefully acknowledged. We are grateful to dr. Goran Anačkov, Department of Biology and Ecology, Faculty of Natural Sciences, University of Novi Sad, for support in term of confirmed and deposited *Morus nigra* L. at the BUNS Herbarium.

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QUANTITATIVE DETERMINATION OF TOTAL ANTHOCYANINS AND FLAVONOIDS IN NATURAL PRODUCTS OBTAINED FROM GRAPES AND MALT

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ABSTRACT: Wines and beers are a rich source of different compounds that show beneficial effects to the human body and their consummation in reasonable amounts can improve health. Among them, the most important are phenolic compounds (nonflavonoids, flavonoids, tannins), which possess antioxidative properties and could be responsible for prevention of many diseases. The aim of this study was to determine the content of total flavonoids, monomeric and total anthocyanins in samples of wines from different grape varieties produced in Serbia and beers produced with the addition of grapes.
Analysis was performed on 14 samples including 5 red wines made from grape varieties from Serbia, one regular beer, and 7 beer samples enriched with different grape varieties and percentage of grape. Monomeric and total anthocyanins were determined spectrofotometrically by the pH differential method, while the total flavonoid content was determined according to the colorimetric Folin-Ciocalteu method after precipitation with formaldehyde.
Anthocyanins were quantified in all analyzed samples, except in regular beer. The highest values were found in wines made from variety Prokupac (270,80 mg/l and 388,79 mg/l malvidine-3-glucoside equivalents, for monomeric and total anthocyanins, respectively). Flavonoids were present in all analyzed samples. Wine samples showed higher content of flavonoids (the highest in Merlot wine) than beer and drinks from beer and grapes. The content of anthocyanins and flavonoids in beer samples enriched with grapes increased with the increase of added amount of grapes. Obtained results showed that relatively high concentrations of flavonoids and anthocyanins are present in wines produced in Serbia. Also, addition of grape in beer increased content of these beneficial phenolic compounds, which can be important factor for quality of a new developed product.
Key words: *anthocyanins, flavonoids , beer, grape*

INTRODUCTION

Beverages such as wines and beer are rich sources of different compounds that show positive effects on human body and their consummation in reasonable amounts can improve health. Many researches have suggested that compounds present in wine may play a protective role in diseases believed to involve, in part, oxidation, such as coronary heart disease, inflammation and carcinogenesis (Lopez-Velez, Martinez-Martinez and del Valle-Ribes, 2003). Also, regular red wine consumption is linked with decrease of cardiovascular mortality in some populations despite high intake of food rich in saturated fats ("French paradox") (Renaud and de Lorgeril, 1992). The reduction in risk of developing diseases for wine consumers is similar to that for consumers of certain fruits, grains and vegetables, which are core components of a traditional Mediterranean diet. The similarity between wine and these foods is the presence of significant amounts of phenolic compounds (Kinsella et al., 1993; Ness and Powles 1997). Phenolic compounds in wines include the non-flavonoid classes of compounds such as the hydroxycinnamates, hydroxybenzoates and the stilbenes, the flavonoid classes of compounds such as flavan-3-ols, flavonols and anthocyanins and

tannins (Stockley and Hoj, 2005). These substances contribute to sensorial characteristics of wine and possess different biological activities. Final composition of phenolic compounds in wine depends on their content in grapes, the extraction parameters, winemaking technology and chemical reaction, which occur during wine aging (Paixao et al., 2007).
Studies conducted in moderate beer drinkers showed a 20–25% reduction in fatality rate from coronary diseases and a reduction by 50% of death factor risk (Bamforth, 2002; Walzl, 2005). These beneficial effects of beer on human health are related to phenolic compounds, particularly to their antioxidant activity (Preedy, 2009). Phenolic compounds identified in beer include phenolic acids, flavonoids, proanthocy- anidins, tannins, and amino phenolic compounds (Gorinstein et al., 2000; Montanari et al.,1999), all of which have been reported to possess antiradical properties as well as other biological effects (Brand-Williams, Cuvelier and Berset, 1995; Gaulejac, Provost and Vivas, 1998). Polyphenols originate from cereal grains (e.g. barley, maize, rice, wheat, sorghum, millet, oat, rye, triticale) and hop; their level in beer is related to malt and hop varieties, malting and brewing processes (Agu, 2002; Derdelinckx, 2008; Qingming et al., 2010). These substances have also important technological role in beer: they are involved in foam maintenance, physical and chemical stability and shelf life of beer.
The aim of this study was to determine the content of total flavonoids, monomeric and total anthocyanins in samples of wines from different grape varieties produced in Serbia and beers produced with the addition of grapes.

MATERIAL AND METHODS

Analysis was performed on 14 samples including 6 red wines, 7 beer samples with different percentage of grape and regular beer. All samples were produced at the enological station Radmilovac of Faculty of Agriculture, Zemun, Belgrade. Wines were made from varieties Merlot (M), Cabernet Sauvignon (CS), Prokupac (P), and Pinot Noir (PN). Also, blackberry wine was analyzed. During production of beer samples appropriate amounts Prokupac, Cabernet Sauvignon and Pinot Noir grape pomace (20, 30 or 50%) were added.
Analysis was performed using Agilent 8453 UV-Visible Spectroscopy System (Germany). Folin Ciocalteu's phenol reagent was purchased from (Fluka Biochemica, Switzerland). Substances and reagents used in experiment were potassium chloride (Zorka Pharma, Serbia), cc hydrochloric acid (Lach-ner, Czech Republic), sodium acetate (Poch, Poland), ethanol 96% (Zorka Pharma, Serbia), formaldehyde 37% (Carbo-Erba, Italy), phloroglucinol-2-hydrate (Centrohem, Serbia), sodium carbonate (Sinex laboratory, Serbia) and gallic acid (Alfa Aesar, England).

Total anthocyanin content

The determination of the total anthocyanins was conducted by the method proposed by Di Stefano, Cravero and Gentilini (1989). The samples were diluted with a solution consisting of 70/30/1 (v/v/v) ethanol/water/HCl (concentrated) and the absorbance was measured at 540 nm. The total anthocyanin contents were expressed as malvidin-3-glucoside equivalents using the following equation:
$TA_5 = A_{540nm} \times 16.7 \times d$
d-dilution

Monomeric anthocyanin content

Monomeric anthocyanins were determined spectrofotometrically by the pH differential method proposed by Lee, Durst and Wrolstad (2005). The samples were diluted with pH 1.0 buffer and pH 4.5 buffer and apsorbance was measured at 520nm and 700 nm against distilled water as blank. The monomeric anthocyanin contents were expressed as malvidin-3-glucoside equivalents using the following equation:

$$MA = \frac{A \cdot M \cdot d \cdot 10^3}{e \cdot l}$$

$A=(A_{520}-A_{700})pH1-(A_{520}-A_{700})pH4.5$
M-molar mass of malvidin-3-glucoside (463.3 g/mol)
d-dilution
ε- molar extinction coefficient (28000 l/mol*cm)
10^3-factor for conversion of g to mg

Total flavonoid content
To 5 ml of sample, 2.5 ml of HCl:H_2O (1:4, v/v) solution and 2.5 ml of 37% formaldehyde were added and left for 2 h. Then, 2 ml of phloroglucinol (10 mg/ml) is mixed into samples and left for 24h at room temperature. Supernatant was filtered through 0,45 μm filter and total phenolic content was determined by the Folin-Ciocalteu's (FC) method using gallic acid as standard (Woraratphoka, Intarapichet and Indrapichate, 2007). Volume of 100 μl of sample or gallic acid standard was mixed with 500 μl of Folin reagent and left for 6 minutes. After that, 400 μl of 7.5% Na_2CO_3 was added, vortexed, and left in dark for 2h. The absorbance of the standards and samples was measured at 740 nm. The amount of flavonoid was calculated as the differences between total phenols and non-flavonoids in samples. The flavonoid content was expressed in mg/l gallic acid equivalents.

RESULTS AND DISSCUSSION

The total and monomeric anthocyanin content in analyzed samples expressed as malvidin-3-glucoside equivalents is shown in Table 1. Total anthocyanins were detected in range from 34.19 to 388.79 mg/l, while monomeric were in range from 39.82 to 270.80 mg/l. The highest content of total as well as monomeric anthocyanins was detected in wine sample made from cultivar Prokupac (inox). Anthocyanins were not detected in regular beer sample. As expected, the highest content of anthocyanins was observed in wine samples, because of high content of these compounds in grapes which contribute to wine color.
Observed variations in total and monomeric anthocyanin content in wine samples can be explained with the influence of cultivar, winemaking technology as well as wine maturity (younger wines have more monomeric anthocyanins) (Degenhadt, 2000). Also, monomeric anthocyanins are involved in hydrolysis, oxidation and polymerization reactions in wine, which can lead to decrease of their concentration. Total anthocyanin content in analyzed wines was in range from 180.18 to 388.79 mg/l, which corroborates with previously published results for wines from Macedonia (Ivanova, Stefova and Vojoski, 2009). The highest content was found for cultivar Prokupac, while the lowest was in wines made from Pinot Noir. The highest monomeric anthocyanin content was also found in cultivar Prokupac, which is not in concordance with results for Serbian commercial wines where the highest content was found for cultivar Cabernet Sauvignon (Radovanović, Radovanović and Jovančićević, 2009).
In beer samples enriched with grape pomace, it is noticeable that increase of grape pomace quantity lead to increase of anthocyanin content. The highest content of anthocyanins was measured in beer sample with 50% Cabernet Sauvignon, while the lowest was in beer with 20% of Pinot Noir. Although variety Prokupac showed the highest content of anthocyanins in wine samples, the same effect was not observed in beer samples, probably because of different extraction of these compounds from varieties. Also, as it can be seen from Figure 1, comparison between wine and beer made with varieties Cabernet Sauvignon and Pinot Noir showed a proportional increase of anthocyanin content. Additionally, quantity of extracted monomeric and total anthocyanins from Pinot Noir pomace in beer is the same (19% and 44%, respectively) when compared with anthocyanin content in Pinot Noir wine.

Table 1. Total and monomeric anthocyanin content of analyzed samples

Wine and beer samples	MA	TA
Pinot Noir wine	109.68±0.67	180.18±0.49
Cabernet Sauvignon wine	157.46±0.22	250.69±0.54
Merlot wine	130.39±0.57	226.11±0.67
Prokupac inox wine	270.80±0.33	388.79±0.66
Prokupac baric wine	222.49±0.14	349.33±0.71
Blackberry wine	160.64±0.38	245.89±0.52
Beer with 20 % Prokupac	39.82±0.09	56.02±0.49
Beer with 30 % Prokupac	39.99±0.26	57.24±0.61
Beer with 20 % Prokupac (II)	59.23±0.73	100.33±0.29
Beer with 20 % Cabernet	42.51±0.81	70.35±0.28
Beer with 50 % Cabernet	105.26±0.39	157.52±0.34
Beer with 20 % Pinot Noir	20.54±0.21	34.19±0.20
Beer with 50 % Pinot Noir	47.99±0.33	79.99±0.47
Beer	0	0

Results are given as mean ± standard deviation (n = 3)

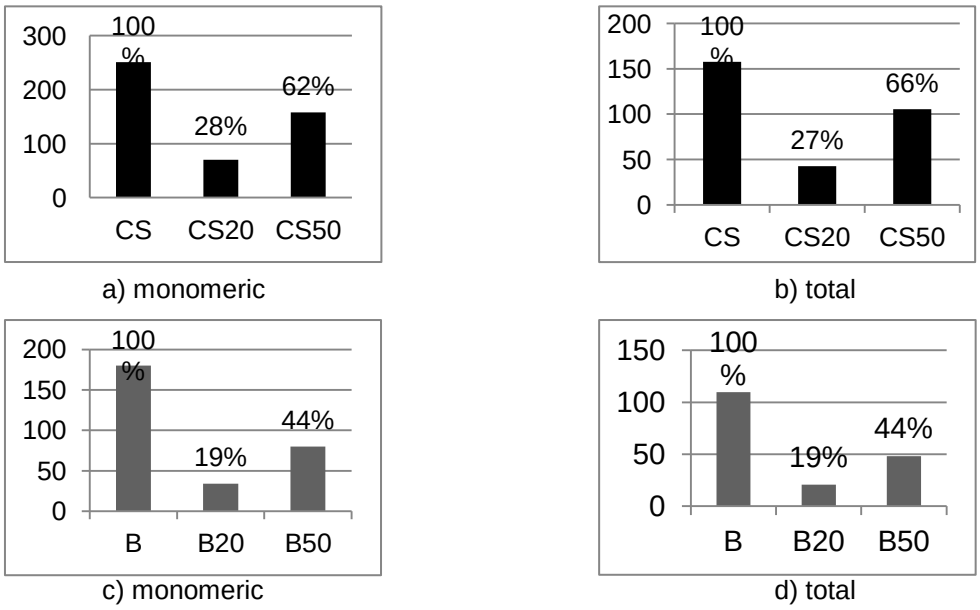

Figure 1. Percentage of extracted monomeric (a,c) and total anthocyanins (b,d) in wine and beer enriched with grape from varieties Cabernet Sauvignon (a,b) and Pinot Noir (c,d)
CS-Cabernet Sauvignon wine, CS20, CS50-beer with 20%, 50% Cabernet Sauvignon grape pomace
B-Pinot Noir wine, B20, B50-beer with 20%, 50% Pinot Noir grape pomace

Table 2. Total flavonoid content of analyzed samples

Wine and beer samples	Total flavonoid content
Pinot Noir wine	1665.39±0.50
Cabernet Sauvignon wine	1687.66±0.35
Merlot wine	1829.22±0.27
Prokupac inox wine	887.94±0.34
Prokupac baric wine	805.41±0.62
Blackberry wine	651.62±0.95
Beer with 20 % Prokupac	284.85±0.40
Beer with 30 % Prokupac	341.88±0.20
Beer with 20 % Prokupac (II)	361.35±0.13
Beer with 20 % Cabernet	172.84±0.17
Beer with 50 % Cabernet	298.33±0.22
Beer with 20 % Pinot Noir	161.85±0.20
Beer with 50 % Pinot Noir	230.60±0.89
Beer	122.07±0.63

Results are given as mean ± standard deviation (n = 3)

The total flavonoid content was in range from 122.07 to 1892.22 mg/l GAE. The highest flavonoid contents were measured in wines from cultivar Merlot, Cabernet Sauvignon and Pinot Noir. The lowest was detected in regular beer (Table 2). Some previously published results for flavonoid content in wine were from 686 to 1104 mg/l GAE (Ivanova, Stefova, Chinnici, 2010), which is lower than our results and could be due to influence of different factors, such as variety, vintage and winemaking technology. In beverages made from grape and beer flavonoid content was from 161.85 to 361.35 mg/l GAE. The highest content of flavonoids was in samples produced with Prokupac grape pomace. Similar to anthocyanis, flavonoid content in beer samples increased with increase of added grape pomace quantity.

CONCLUSIONS

Anthocyanin and flavonoid content was determined in wines and beer enriched with grape. The highest content of monomeric and total anthocyanins was quantified in red wines. The biggest values were found in wines made from variety Prokupac (270,80 mg/l and 388,79 mg/l malvidine-3-glucoside equivalents, respectively). Wine samples also showed higher content of flavonoids (the highest in the Merlot wine) than beer and drinks from beer and grapes. The content of anthocyanins and flavonoids in samples of beer enriched with grapes increased with the increase of added amount of grapes in them. These results show that relatively high concentrations of flavonoids and anthocyanins are present in wines produced in samples from Serbia. Also, it is noticeable that grape enriched beer has increased content of these beneficial compounds, which can improve quality of new developed product.

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ACRYLAMIDE FORMATION IN COOKIES WITH COFFEE EXTRACTS

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ABSTRACT: The aim of this study was the development of improved cereal products with various types of extracts prepared from coffee and the evaluation of quality and safety of these novel products. In this study, the addition to cookies of freeze-dried aqueous and ethanol soluble fractions, a microwave-assisted extract, and an oil extract recovered from the coffee residue, was evaluated on acrylamide content and preliminary sensory acceptance by consumers. High and low molecular weight fractions obtained from water soluble coffee were separated using ultrafiltration. All kinds of coffee extracts and coffee oil were incorporated into the formulation of cookies in various concentrations (from 0.1 % up to 5 %). None of coffee extracts increased acrylamide formation. Moreover, in the case of the aqueous fractions, acrylamide elimination up to 10 % was observed (from 223 ± 6 µg/kg to 198 ± 3 µg/kg) which is important from the safety point of view. It was observed that the highest intensity of coffee flavour was in cookies with ethanol soluble fraction in comparison to other coffee extracts.
Key words: *acrylamide, coffee extracts, functional cereal products, quality and safety*

INTRODUCTION

Coffee polysaccharides, which are galactomannans and arabinogalactans (1), present immunostimulatory activity (2,3) and are associated with a decreased risk of a number of disorders including colorectal cancer, constipation, diverticulosis, cardiovascular diseases and type II diabetes. Phenolic compounds, namely chlorogenic and caffeic acids, as well as melanoidins that are formed during roasting, are known for their antioxidant properties (4). On the other hand, some of these components are also potential reagents in the process of Maillard reaction in which acrylamide, a known health-hazardous compound, is naturally formed during baking of cereal products in significant levels (5). Since the presence of acrylamide in heated starch-based foods was discovered in 2002, extensive efforts have been undertaken to investigate tools that may lead to a reduction of acrylamide during food processing. For that reason a special attention is focused on the impact of coffee extracts potential on acrylamide mitigation due to a high content of compounds with antioxidant capacity such as phenolic compounds and melanoidins that were reported to be able to eliminate acrylamide formation (e.g. 7-8 etc.). Evaluation of risk-benefit showed an importance also due to an observation of some health benefit ingredients such as fruit fibre that dramatically increased acrylamide content that was reported before (9). A presented pilot study of bilateral cooperation is aimed at preparing of cereal products with health benefits and regulated safety hazard risk from acrylamide formation point of view.

MATERIAL AND METHODS

Coffee extracts

Five coffee samples were produced in laboratories of University of Aveiro from instant coffee/ coffee residue:

1. Ethanol soluble fraction (EtSn)
2. Low-molecular weight aqueous fraction (LMWaq)
3. High-molecular aqueous fraction (HMWaq)
4. Microwave extract (ME)
5. Oil extract (OE)

For ethanol and aqueous fractions, soluble instant coffee was dissolved in water (80 °C) and precipitated in ethanol. The supernatant material was recovered as the ethanol soluble fraction (ETSn). The ethanol precipitated material was recovered, re-dissolved in water and submitted to a ultrafiltration separation process with a stirred ultrafiltration cell (Millipore Corp., MA, USA) using a 5000 Da filter, from which resulted the low molecular weight aqueous fraction (LMWaq) and high molecular weight aqueous fraction (HMWaq). The microwave extract (ME) was obtained from 20 g of defatted wet coffee residue (≅ 60% w/w of water) suspended in 60 mL of water in a high pressure reactor of 100 mL. The mixture was maintained in homogeneous conditions by mixing with a magnetic stirrer. Microwave power was adjusted to attain 170°C in 2 min, and maintain it for 5 min more. All samples were freeze-dried to powder consistence. Coffee oil (OE) was extracted from pre-dried coffee residues using the Soxhlet method.

Cookies preparation

Coffee extracts were applied to cookie dough in concentration range of 0.1 – 5 % and 10% (w/w), respectively, regarding the extract type in relation to sensory preferences of final product. Dough was prepared from wheat flour, shortening, sucrose, brown sugar, deionized water, salt, non-fat powdered milk, high-fructose corn syrup and raising agents according the standard prescript of AACC Method No. 10-54 (10) using a homogenizer (KitchenAid, USA). Samples of cookies were round shaped to diameter of 6 mm and height of 8 mm and baked at 205 °C for 11 min.

Acrylamide analysis

Final acrylamide content in cookies was analyzed by LC/ESI-MS-MS method using an HPLC system 1200 series (Agilent Technologies, USA) coupled with an Agilent 6410 Triple Quad detector equipped with an ESI interface according to the procedure published in detail before (11).

Sensory evaluation and colour analysis

The impact of coffee extracts and coffee oil on organoleptic properties was evaluated by the panel of 3 dependant judges and 2 - 5 independent assessors with the aim to set the acceptable addition of each extract to cookies. Properties such as colour, texture, appearance, flavour (special descriptors: an intensity of coffee, an intensity of caramel) and taste (special descriptors: an intensity of coffee, an intensity of bitter, an intensity of sweet and caramel) were consider in to account with correlation of consumer preferences.

The colour of a top and a bottom of cookies was measured directly using a Color i5 Spectrophotometer (X-Rite, Germany) at D65/10° UV selection. To distinguish colour changes visual limits in CIELab system were set to: DL=+2/-2, Da=+2/-2, Db=+2/-2 by an X-RiteColor MASTER software.

RESULTS AND DISCUSSION

Acrylamide formation

A presented pilot study was aimed at the development of a new cereal product with functional properties conferred by the addition of various coffee extracts rich in antioxidants. It was proved out that the incorporation of coffee extract represented a safe model of functional food in context with acrylamide formation during thermal processing; moreover some type of extracts resulted in moderate acrylamide mitigation (Fig. 1).

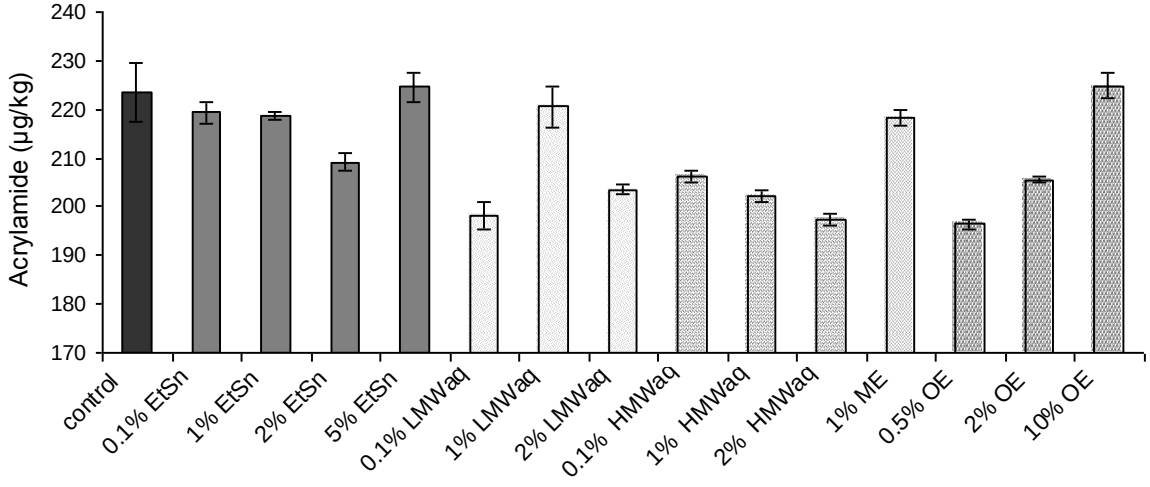

Fig. 1 Influence of coffee extracts addition on acrylamide content in cookies

Approximately a 10% decrease was observed using a LMWaq fraction and a HMWaq in concentration up to 2 % (w/w). The addition of coffee oil also reduced acrylamide content, but only in lower concentration (up to 2%), nevertheless in higher concentration the impact of coffee oil on acrylamide mitigation was not observed (Fig. 1). The addition of a microwave extract in concentration of 1 % (w/w) was not effective.

Sensory evaluation

In case of novel food product development, a sensory evaluation plays an important role. The impact of extracts on organoleptic properties of cookies such as colour, texture, flavour and taste was different. Colour of cookies was measured spectrophotometrically on a top and a bottom of samples and compared with a standard using a CIELab colour system. It could be summarised that LMWaq and HMWaq fractions had the most significant impact on colour. Nevertheless, the impact on the top and the bottom was different. The top of cookies was darker, less yellow and less red; and the bottom, vice versa, lighter, more yellow and more red, especially using the HMWaq fraction. Other extracts, mainly ME and EtSn ones had a small influence on colour of cookies.

According to the preference test, the LMWaq fraction and also EtSn fractions were evaluated in a positive way with the best score of 2 % (w/w) of EtSn (the maximal total score was 3 points) due to the highest harmony in sweet and bitter taste and crunchy delicate soft texture. The addition of 5 % (w/w) of EtSn fraction influenced the intensity of coffee aroma in the highest extends that led to the increase of the total score from 2.0 to 2.5 points in comparison to the control sample. The addition of coffee oil was acceptable up to 2 % (w/w) addition (2 points in total score). The higher content of coffee oil deteriorated preferences of cookies (1 point in total score) due to off-flavour (burned, popcorn-like). The HMWaq fraction was also evaluated in a positive way for their sweet-chocolate taste (2 points). HMWaq fraction did not give coffee smell or taste at all.

Tab. 1 Sensory evaluation score of cookies with coffee extracts

Coffee extract type	Concentration of extract (% w/w)	**Total score** (1- bad quality, 2 – standard/good quality, 3 – improved quality)
EtSn	0.1	2
	1	2.5
	2	2.5
	5	2
LMWaq	0.1	2
	1	2
	2	3
	5	3
HMWaq	0.1	2
	1	2
	2	2
ME	1	2
OE	1	2
	2	2
	10	1

CONCLUSIONS

The novel type of food product was prepared using various coffee extracts without a negative impact on acrylamide formation; moreover with a mitigation potential that is proposed for further research. It was proved out that the most promising was the addition of aqueous coffee extracts (both low and high molecular weight aqueous fractions) that also improved organoleptic properties of cookies.

ACKNOWLEDGEMENTS

This contribution is the result of the project implementation "A strategy of acrylamide elimination in food technology processing" supported by the Research & Development Operational Programme funded by the ERDF. This work was also supported by the Slovak Research and Development Agency under the contracts No. LPP 0310-09 and SK-PT-0027-10. This work is also the result from the a Slovak-Portugal collaboration entitled “Cooperation at the development of improved functional cereal products” established between the VUP Food Research Institute, Department of Chemistry and Food Analysis, Bratislava, Slovak and the Research Unit 62/94 QOPNA (project PEst-C/QUI/UI0062/2011), Department of Chemistry, University of Aveiro, Aveiro, Portugal. The portuguese team was funded by the FCT. Cláudia Passos was supported by a post-doc grant by FCT (SFRH/BDP/65718/2009).

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PHYSICAL PROPERTIES OF FUNCTIONAL COFFEE BEVERAGES – INULIN AND OLIGOFRUCTOSE ENRICHMENT

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ABSTRACT: Coffee is a wide spread commodity nowadays, which millions of people consume daily. The idea of this work was to enhance the nutritional composition of coffee by inulin and oligofructose enrichment. Inulin and oligofructose are known for its prebiotic properties and have many health benefits. Powdered mixture of inulin and oligofructose was added to three most common coffee beverage types: milled coffee, instant coffee and cappuccino. Physical properties of the control samples (without the addition of functional ingredients) and the samples containing the functional ingredients were monitored. Particle size, moisture content, colour, bulk density, cohesion index, powder flow speed dependency profiles and caking profiles were determined in order to get an insight on the changes caused by the addition of the functional powdered ingredient. Results showed that the addition of inulin and oligofructose lowered the cohesion index of the functional mixtures for all three tested coffee preparations. The addition of the functional ingredient also caused a decrease in cake strength in the case of instant and milled coffee. Colour change was visible for all samples based on the calculated ΔE values (ΔE = 4.64 for cappuccino, ΔE = 5.48 for instant coffee and ΔE = 1.01 for milled coffee). A slight decrease was also detected in Hausner ratio values. Furthermore, moisture content had a significant influence ($p<0.05$) on the cohesion index, caking profiles and Hausner ratio, while particle size strongly influenced cake height ratios recorded during the caking test. These findings suggest that the addition of functional ingredients does not lead to a deterioration of physical properties, and that functional coffee beverages represent an interesting aspect of future research.
Key words: *coffee, enrichment, physical properties*

INTRODUCTION

Coffee, as one of the widest spread beverages nowadays, is known for some of its health benefits, which include the high levels of functional ingredients, such as antioxidants (Brezova et al., 2009). While the health benefits of antioxidants (e.g. polyphenolic compounds) contained in coffee are well described (Bonita et al., 2007), based on the growing market for functional products, there is a growing need for development of enriched coffee beverages, which would contain other functional ingredients such as vitamins, minerals, long chain carbohydrates or even polyunsaturated long chain fatty acids. These coffee beverages would be aimed to ensure at least part of the nutritional requirements of millions consumers around the world which enjoy their coffee on daily basis. So far, there have been several attempts to patent nutritionally enriched coffee beverages (Sartorio et al., 1999, Atkinson et al., 2001), which mostly contain vitamin and mineral premixes. On the other hand, inulin and oligofructose, long chain carbohydrates, as functional ingredients have many health benefits, such as improvement of the bowel health and promoting of the probiotic bacteria growth (Bonnema et al., 2010). Chicory roots, from which inulin is made, is already used in coffee industry for production of e.g. coffee beverages for diabetics (Alves et al., 2010). However, its usage in creating functional milled of instant coffee products has not been recorded yet. Since three most widely used coffee preparations (milled coffee, instant coffee and cappuccino preparations) are powdered products, it is expected that the addition of inulin and oligofructose powder by dry mixing the ingredients, would affect the physical properties of the mixes. This paper aims to detect the overall differences and changes in physical properties of three powdered coffee products (milled coffee, instant coffee and cappuccino) caused by the addition of inulin and oligofructose as functional ingredients.

MATERIALS AND METODS

Materials

Ground coffee, instant coffee and cappuccino products were supplied by Franck, Croatia. Inulin and oligofructose premix was obtained from a local manufacturer (Magdis d.o.o., Croatia). The samples were prepared as follows: three mixtures of 250 grams of coffee (ground, instant and cappuccino) and 12.5 grams of the inulin/oligofructose premix were mixed for 10 minutes in a Turbula mixer (Willy Bachoffen Maschienenfabrik, Switzerland) to obtain a homogenous mixture. Immediately after preparation, moisture content, colour and physical properties were analysed.

Particle size

Particle size of the samples was analysed by laser diffraction using Mastersizer 2000 (Malvern Instruments, UK), equipped with Scirocco 2000 dry dispersion unit. Particle size analysis of instant coffee and cappuccino powders was conducted at following instrument settings: 1 bar air pressure, 50% feed rate and laser obscuration limits ranging from 2 – 6%. Ground coffee samples were dried using an oven dryer for 3 hours at 105 °C prior to measurement, which was conducted at 2-6% laser obscuration, 4 bar air pressure and 100% feed rate, to ensure the optimal flow of the sample through the measurement unit. Results were recorded as d (0.1), d (0.5), d (0.9), D [3,2] and D [4,3]. The measurements were done in triplicate.

Bulk density and Hausner ratio

Bulk density was determined based on the method by Haugaard Sorensen et al (1978). The powder was poured freely into a container, weight of the powder in the container was recorded and the powder was tapped 1250 times using a laboratory made jolting volumeter. Hausner ratio was calculated by dividing the 1250 times tapped density with poured bulk density. All measurements were done in triplicate.

Moisture content

Moisture content of the samples was determined by drying at 105 °C for 3 hours in an oven dryer. The samples were weighed on an analytical balance (Sartorius, Germany) prior to drying and after drying. The difference in weight before and after drying was recorded as the mass of the water contained in the sample. Measurements were done in triplicate.

Colour

Colour measurements were performed using a CM-700d spectro photometer (Konica Minolta Sensing INC., Japan). Prior to measurement white calibration was conducted using a calibration plate supplied by the manufacturer of the instrument. Brightness (L), redness (a) and yellowness (b) were recorded.. The total colour difference (ΔE), as a combination of *L*, *a* and *b* values, also calculated to get a comprehensive insight of the changes in colour caused by the addition of functional ingredients. Samples without the addition of inulin and oligofructose were considered reference samples in ΔE calculation. All the measurements were done in triplicate.

Flow properties

Flow properties of the samples were tested using TA.HDPlus Powder Flow Analyser coupled with the TA.HDPlus Texture Analyser (Stable Micro Systems, Surrey, UK). Three tests were performed: quick test (cohesion assessment), powder flow speed dependency test (PFSD) and caking test. A fixed sample volume (160 mL) was poured into a glass container prior to testing. Prior to testing, the instrument performed a 2 cycle sample preparation step, for each test. Cohesion property was assessed by quick test, with the blade moving upwards through the powder column at the tip speed of 50 mms^{-1}. Cohesion coefficient was calculated by integrating the negative area under the force/ distance curve, and the cohesion index was calculated by dividing cohesion coefficient with sample weight. Powder Flow Speed

Dependency test (PFSD) started with two conditioning cycles which were followed by cycles run at a tip speed of 10 mms^{-1}, 20mms^{-1}, 50 mms^{-1}, 100 mms^{-1} and two final cycles at 10 mms^{-1}. The area under the positive part of the curve, which is the work of compaction, was calculated using Texture Exponent 32 software (Stable Micro Systems, Surrey, United Kingdom). During caking test, the blade levelled the top of the powder column and measured the height of the column, after which it moved down through the column at a tip speed of 20 mms^{-1} and compacted the powder to a pre-defined force (usually 750 g). When the blade reached the required force it sliced up through the powder at 10 mms^{-1} and repeated the compaction cycle four more times. At the beginning of every cycle the blade measured the height of the column and the height of the powder cake was recorded when the target force was reached. The fifth time the target force was reached the blade cut through the formed powder cake at the bottom of the vessel and measured the force required to perform the task. This force was recorded as the cake strength and represented the work required to cut the cake (gmm). Cake height ratio (current cycle cake height divided by initial column height) was recorded to give information about the settlement and compaction of the powder column.

RESULTS AND DISCUSSION

Results for particle size distribution, moisture content and bulk density are shown in Table 1.

Table 1..Moisture content, particle size distribution and bulk density of the samples

Sample	Moisture content [% w/w]	Particle size distribution [µm]					Bulk density [kgm^{-3}]		
		d (0.1)	d (0.5)	d (0.9)	D [2,3]	D [4,3]	Poured	Tapped	Hausner ratio [/]
Ground–control	1.01± 0.01	39.84± 0.72	272.39± 1.57	518.08± 2.79	110.30± 1.39	283.89± 1.95	327.37± 2.77	481.42± 2.44	1.47
Ground-inulin	1.52± 0.02	42.55± 0.39	263.49± 0.48	516.18± 0.68	112.10± 0.69	278.00± 0.59	332.24± 4.33	482.91± 6.98	1.45
Instant-control	4.93± 0.02	24.72± 0.20	150.24± 6.63	577.24± 34.28	57.55± 0.90	229.62± 3.47	360.49± 4.12	455.16± 7.43	1.26
Instant-inulin	4.81± 0.05	24.78± 0.77	127.18± 6.64	495.00± 29.62	53.20± 3.54	198.67± 1.19	369.24± 3.21	456.99± 2.44	1.24
Cappuccino-control	1.44± 0.02	39.96± 1.25	258.49± 1.10	674.61± 1.23	70.63± 0.53	314.50± 0.96	295.39± 3.86	376.77± 4.65	1.28
Cappuccino-inulin	1.82± 0.03	32.38± 2.51	232.36± 4.95	578.43± 11.92	66.80± 0.20	277.06± 5.72	290.54± 2.98	352.60± 3.16	1.21

As shown in Table 1, addition of inulin/oligofructose premix caused a drop in d(0.1), d(0.5), d(0.9), D[3,2] and D[4,3] values in most cases, with an exception of milled coffee d(0.1) and D[3,2] which exhibited a slight insignificant increase. This increase could be of technical nature. During the measurement it was impossible to achieve stable laser obscuration due to the nature of the ground coffee sample. This might have caused slight discrepancies in the results. Ground coffee showed the highest d(0.1), d(0.5) and D[2,3] values, while instant coffee showed the highest d(0.9) and D[4,3] values. Instant coffee samples had the highest moisture content values, which represents a logical finding if we take into consideration the production process of instant coffee. Addition of inulin caused a slight rise in the moisture content of ground coffee and cappuccino and a slight decrease in the moisture content of instant coffee. Differences in bulk densities of control and enriched samples were slight and insignificant. Furthermore, a slight decrease of the Hr values was detected for enriched samples.

Cohesion indexes of the powders assessed by powder rheometer are shown in Figure 1.

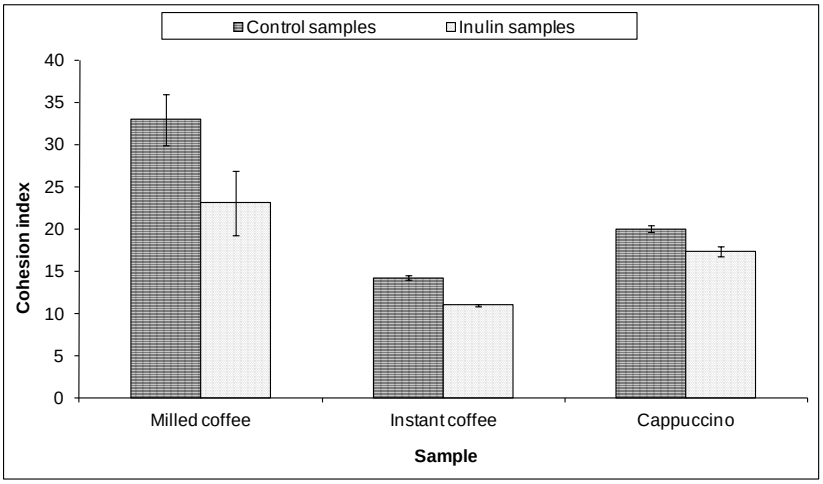

Figure 1. Comparison of the cohesion indexes of the samples

Addition of inulin/oligofructose premix caused a drop in the cohesion index values. The highest drop was detected for ground coffee samples, followed by instant coffee and cappuccino (Fig.1). Based on the cohesion index, both ground coffee samples were categorized as hardened, extremely cohesive, instant control sample as cohesive, enriched instant coffee sample as free flowing, cappuccino control sample as hardened/extremely cohesive and enriched cappuccino sample as very cohesive. In general, instant agglomerated coffee powders show lower cohesion index than ground coffee or cappuccino, whose high cohesion index is due to the presence of small particles, sugars and skim milk powder, which is known to have poor flow properties (Ilari, 2002).
Powder flow speed dependency test resulted in compaction coefficient profiles shown in Figure 2.

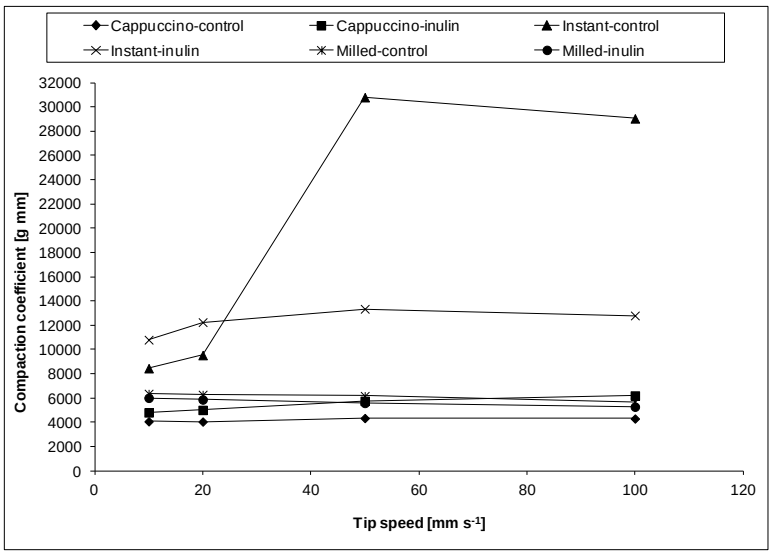

Figure 2. Compaction coefficient profiles of tested samples

Cappuccino showed the lowest compaction coefficient values, followed by ground and instant coffee. In the case of cappuccino, the addition of inulin/oligofructose premix caused a slight increase in compaction coefficient values, while its addition to ground and instant coffee caused a drop in compaction coefficient values. Compaction coefficient values which increased with a rise in tip speed, as detected for instant coffee samples, also meant that the sample flowed harder at higher tip speeds. This deterioration in flow properties was due to agglomerate brakeage which occurred at high tip speeds which resulted in a larger percentage of small diameter particles in the sample. Both ground coffee samples showed a drop of compaction coefficient values with increasing flow speed, which meant they flowed

better at higher conveying speeds. Inulin/oligofructose enriched cappuccino sample showed an increase in compaction coefficient values with higher tip speed, indicating that its flow is harder at higher speeds. The only sample showing a relatively unchanging compaction coefficient profile is cappuccino control sample, indicating it was flow speed independent.
Results of the caking test, recorded as the dependence of cake height ratio towards cycle number, are shown in Figure 3.

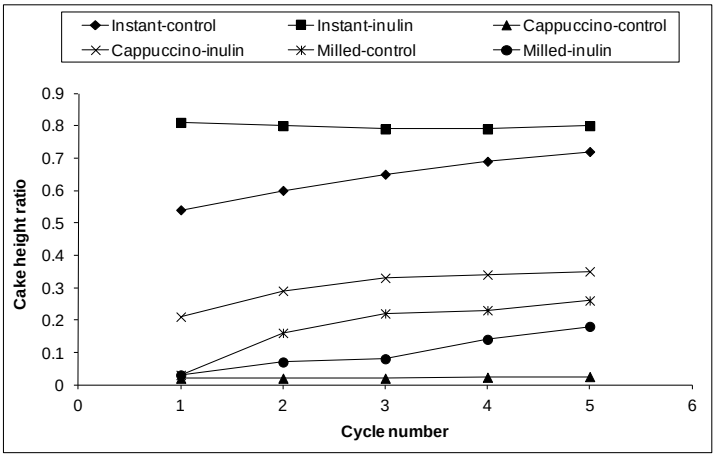

Figure 3. Caking profiles of the tested samples

As shown in Fig.3. instant coffee samples showed the highest cake height ratio values. Instant coffee and cappuccino samples with the addition of inulin and oligofructose showed higher cake height ratio values in comparison to their control samples. The opposite trend was detected for ground coffee – enrichment lead to lower cake height ratio values. Furthermore, cappuccino control sample and inulin enriched instant coffee samples showed an unchanging cake height ratio through out all five caking samples, while all the other samples showed an increase in cake height ratio, which meant they were susceptible to caking. Cake strength values were also recorded during the caking test: for instant-control 4683.93 gmm, instant-inulin 3828.50 gmm, ground-control 2105.93 gmm, ground-inulin 1160.04 gmm, cappuccino control 125.33 gmm and for cappuccino-inulin 432.89 gmm. According to these results, the addition of inulin/oligofructose premix caused a drop in cake strength, which is favourable in transport and handling, while the addition of inulin/oligofructose premix caused an unfavourable increase in cappuccino cake strength. However, since cappuccino already showed by far the lowest cake strength value, enrichment cannot be considered as deteriorating for the cappuccino flow properties.

Table 2. Colour of the analysed samples

Sample	L	a	b	ΔE
Ground-control	28.23±0.04	11.25±0.04	19.29±0.01	1.01
Ground-inulin	28.67±0.01	12.16±0.01	19.22±0.01	
Instant-control	29.78±0.17	13.81±0.08	23.47±0.32	5.48
Instant-inulin	33.07±0.20	12.12±0.28	19.42±0.57	
Cappuccino-control	79.47±0.04	2.21±0.01	7.95±0.06	4.64
Cappuccino-inulin	83.99±0.06	1.55±0.01	7.14±0.02	

As shown in Table 2., L values (brightness) were the highest for cappuccino samples, followed by instant and ground coffee. The addition of inuline/oligofructose premix caused an increase in brightnes for all the samples. Also, the L value was affected the strongest by inulin addition, which represents a logical finding considering the white colour of inuline/oligofructose premix. Redness (a) and yellowness (b) decreased with the addition of inulin, with the exception of redness (a) value for enriched ground coffee. The greatest total

colour difference was detected for instant coffee, followed by cappuccino and ground coffee. These results were somewhat surprising since it was expected that the great colour difference between ground coffee and inulin would result in highest ΔE values. However, it appeared that the darkness of ground coffee particles became dominant over the white inulin particles, thus diminishing the total colour deference between the standard and enriched sample.

Statistical analysis of the overall physical properties of the samples showed the following: significant ($p<0.05$) positive correlations were found between control and enriched samples. Median diameter [d (0.5)] significantly affected cohesion index, compaction coefficient at 10 and 20 mms^{-1}, all cake height ratios and cake strength values. Sauter diameter (D[4,3]) had a significant influence on cohesion index (R=-0.88, p=0.019), while D[4,3] influenced poured bulk density, compaction coefficients at 10 and 20 mms^{-1}, all cake height ratios and cake strength values. Moisture content affected the following properties: compaction coefficients al all four tested tip speeds (with R values ranging from 0.84 for 500 mms^{-1} (p=0.036) cycle to 0.89 (p=0.016) for 20 mms^{-1}), all cake height ratios and cake strength values (R=0.7, p=0.024). Moisture significantly affected d (0.1) (R=-0.93, p=0.007), d (0.5) (R=-0.98, p=0.001) and D[4,3] (R=0.91, p=0.012). Furthermore, a strong correlation was also found between bulk density and all colour parameters, which meant that the way the particles were packed together played an important role in colour of the mixtures. Bulk density showed strong correlation with cohesion index (R=-0.92, p=0.01), d(0.5) (R=-0.83, p=0.041) and D[3,2]. Significant ($p<0.05$) positive correlation was found between compaction coefficient values at 10 and 200 mms^{-1}, all cake height ratios and cake strength values.

CONCLUSIONS

Addition of inulin/oligofructose premix lowered the cohesion index and Hr values of the functional mixtures for all three tested coffee preparations. The addition of the functional ingredient also caused a decrease in cake strength in the case of instant and milled coffee. Furthermore, moisture content had a significant influence ($p<0.05$) on the cohesion index, caking profiles and Hausner ratio, while particle size strongly influenced cake height ratios recorded during the caking test. Colour change was visible for all samples based on the calculated ΔE values. These findings suggest that the addition of functional ingredients does not lead to a deterioration of physical properties.

ACKNOWLEDGEMENTS

The authors would like to thank “Franck” for supplying the samples used in this research.

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ANALYSIS OF OMEGA-3 FATTY ACIDS CONTENT IN FRESH MARINE FISH

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ABSTRACT: Fish lipids are rich dietary sources of n-3 long-chain polyunsaturated fatty acid (LC-PUFAs). Among n-3 fatty acids, the most important are eicosapentaenoic (20:5 n-3, EPA) and docosahexaenoic (22:6 n-3, DHA) acids which have important roles in the prevention and treatment of different diseases. The aim of this study was to analyze the fatty acid content of fresh marine fish available on the Serbian market. In five species of marine fish (salmon, tuna, mackerel, sardines and hake) the content and composition of fatty acids were analyzed. After lipid extraction, fatty acids were derivatized into volatile methyl-esters. Fatty acids were determined using capillary gas chromatography. Analyses of total lipid content in fresh fish samples and their fatty acid composition revealed that mackerel and sardines were the best dietary sources of LC-PUFAs (4.6 and 4.5 g/100 g, respectively). All analyzed fish samples had significantly higher DHA than EPA content. The highest average EPA content per 100 g of edible fish was observed in samples of sardines (2.0 g). The results showed that the DHA content of sardines, mackerel and salmon were similar - 2.2 g, 2.6 and 2.3 g/ 100 g, respectively. Hake and tuna had significantly lower LC-PUFAs, EPA and DHA contents. Although significant variations in n-3 fatty acids' content were noted among individual fish samples, it could be concluded that fresh marine fish available on Serbian market can be cheap and valuable source of LC-PUFAs in daily diet.
Key words: *n-3 fatty acids, marine fish, analysis*

INTRODUCTION

Marine food and especially marine fish are important sources of long chain polyunsaturated fatty acids (LC-PUFAs). Among the polyunsaturated fatty acids, eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acids (C22:6n3, DHA) are the dominant n-3 fatty acids in marine fish. The importance of n-3 fatty acids in human health is well documented in numerous studies as reviewed by Simopoulos (2011). These fatty acids can be of great importance to humans in prevention of coronary artery disease (Mozaffarian et al, 2005) and other chronic diseases (Simopoulos, 2008). The fat content and the fatty acid composition of fish can vary significanlty. The reasons for fatty acid composition variations are diet, life cycle, temperature, location, gender and environmental conditions (Bandarra et al, 2001; Gockse et al, 2004). Thus, this study was carried out to determine and compare the fatty acid composition in common marine fish available on Serbian market and to evaluate marine fish as sources of dietary n-3 fatty acids.

MATERIAL AND METHODS

Five marine fish species commonly consumed in Serbian diet were chosen and purchased from the local fish market (salmon, tuna, mackerel, sardines and hake). The origin of salmon was Norway (North Sea), while the origin of other four species were Montenegro and Croatia (Adriatic Sea). Skin, head, gills, fins and bones from each fish species were removed, meat was cut into pieces and homogenized. At least three samples from each fish were used for analysis. The total lipids content from each homogenized sample was determined by gravimetric method after Soxhlet extraction. Lipids for further fatty acid analysis were extracted according to the Bligh&Dyer method (1959). The methyl esters of the fatty acids (FAMEs) from the lipid extract were transesterified with HCl in methanol according to the method as described by Ichihara and Fukubayashi (2010). FAMEs were quantified using a

Agilent Technologies 7890A Gas Chromatograph with a flame ionisation detector. Separation of the FAMEs was performed on a 112-88A7, HP-88 capillary column (100 m x 0,25 mm x 0,2 μm) using He as a carrier gas at a flow rate of 105 ml/min. The samples were injected at a starting oven temperature of 175˚C, injector temperature was 250˚C and detector temperature was 280˚C. The oven temperature was programmed to increase from 175˚C to 220˚C at 5˚C/min. Fatty acids were identified by their retention time with reference fatty acid standards (SupelcoTM FAME Mix, USA).

RESULTS AND DISCUSSION

The lipid content was different between analysed fish species and individual samples. The percentage of total lipids was highest in mackerel (14%), while the lowest content was found in tuna (1,2%).
The fatty acid compositions of the investigated fish are presented as mean value in Table 1.

Table 1. Fatty acid composition (% of total amount of fatty acids) of the investigated marine fish

Fatty acid	Hake	Sardine	Mackerel	Salmon	Tuna
14:0	1.4	4.3	3.9	2	0.4
16:0	21.3	21.5	14.5	14.8	17.6
16:1	2.6	5.6	2.3	2	1
18:0	4.9	4.1	3.3	3.3	7.7
18:1n9	10.4	2.8	7.6	22.1	10.6
18:1n7	2.4	3.6	1.4	3.9	1.6
18:2n6	1.2	1.1	2.1	8.2	1.1
18:3n6	0.2	0.3	0.3	0.2	0.1
18:3n3	1.4	1.2	7	2.7	0.3
20:4n6	2	3.5	12	2.1	6.9
20:5n3	8.2	14.7	9.7	6.5	3.2
22:6n3	36.8	27	21.9	21.7	33.3
SFA	27.5	30.4	23.3	20	26.8
MUFA	15.4	12	11.5	27.9	13.4
PUFA	49.7	47.8	52.8	41.4	44.8

SFA - saturated fatty acid; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acid.

The results showed differences in the individual fatty acids among the marine fish species. In all investigated fish the main fatty acids were palmitic acid (C16:0) and DHA. The fatty acids with the highest percentages, next to C16:0 and DHA, were EPA and oleic acid (C18:1n9). These results are in agreement with previous studies (Zlatanos & Laskaridis, 2007; Zlatanos & Sagrados, 1993). The fatty acid composition of marine fish were found to be 20-30.4% saturated (SFA), 11.5-27.9% monounsaturated (MUFAs) and 41.4-52.8% polyunsaturated fatty acids (PUFAs). These results are also in accordance with the literature (Ozogul et al, 2007).

Analyses of total lipid content in fresh fish samples and their fatty acid composition revealed that mackerel and sardines, were the best dietary sources of LC-PUFAs (4.6 and 4.5 g/100 g, respectively). All analyzed fish samples had significantly higher DHA than EPA content. The highest average EPA content per 100 g of edible fish was observed in samples of sardines (2.0 g). The results showed that the DHA content of sardines, mackerel and salmon were similar - 2.2 g, 2.6 and 2.3 g/ 100 g, respectively. Interestingly, tuna and hake had very low DHA and EPA content in comparison with other fish species.
The total n-3 PUFAs and n-6 PUFAs content are shown in Figure 1. Significant variation of n-3 fatty acids' content was noted among individual fish samples. Among the analyzed species, the highest level of n-3 PUFAs was noted in mackerel, whereas hake and tuna had the lowest level of total n-3 fatty acids. In all analysed marine fish species total n-3 PUFAs content were higher than those of n-6 PUFAs. These results are in accordance with previous results (Abdulkadir, 2006).

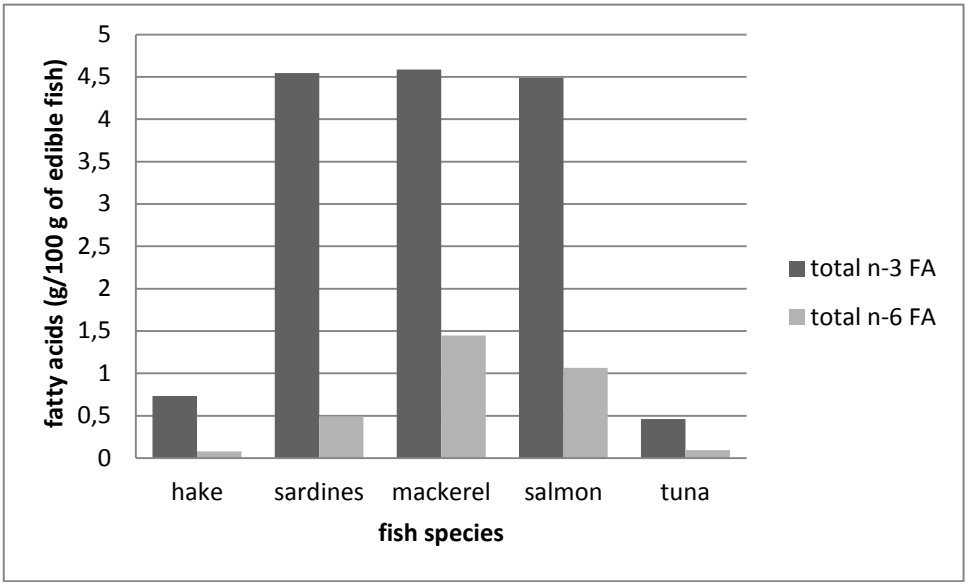

Figure 1. Total n-3 PUFAs and n-6 PUFAs content of the investigated marine fish

CONCLUSIONS

Although there were significant differences in lipid content and fatty acid composition, all five examined marine fish species are rich sources of long-chain n-3 fatty acids, especially DHA. The fish species studied are among the most common found in the Serbian market, and therefore can be regarded as valuable and easily available sources of LC-PUFAs in daily diet.

ACKNOWLEDGEMENTS

This paper is a result of the research within the III-46001 project supported by the Ministry of Education and Science, Republic of Serbia.

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DETERMINATION OF HIDDEN GLUTEN IN FOOD SUPPLEMENTS AND FOODS FOR PARTICULAR NUTRITIONAL USES FROM SERBIAN MARKET

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ABSTRACT: Coeliac disease is characterized by permanent intestinal intolerance to wheat gliadin and related prolamins from barley, rye and oats. The gluten free diet is a life long treatment for coeliac patients. Industrial starch or other ingredients used in manufacturing of dietary products can contain prolamins as impurities. The aim of this work was investigation the presence of gluten in dietary products from the national market: 30 dietary foods for infants and young children and 35 food supplements were analyzed using the Ridascreen Gliadin sandwich R5 enzyme-linked immunosorbent assay with cocktail extraction. The most of the analyzed samples (88%) contained less than limit of quantification of 5 mg/kg for gluten. Gluten content of 5-20 mg/kg was determined in 3 samples. Five of 65 samples (8%) contained mean gluten levels ≥ 20 mg/kg and would not be considered “gluten-free” according to the national Regulation on health safety of dietary products. The systematic control of the presence of gluten in gluten-free dietary products is very important and should be obligatory in order to protect the health of coeliac patients.
Key words: *coeliac disease, gluten, dietary products, food supplements, baby foods*

INTRODUCTION

Coeliac disease (CD), also known as coeliac sprue and gluten sensitive enteropathy, is characterized by permanent intestinal intolerance to wheat gliadin (and related prolamins from barley, rye and oats), which causes damage to small bowel mucosa by an autoimmune mechanism in genetically susceptible individuals (Hill et al., 2005). Epidemiological studies have shown that CD affect approximately 0.5–1.0% of people in developed countries (Catassi and Fasano, 2008) and its prevalence is on the rise (Rubio-Tapia and Murray, 2010). Untreated CD can result in severe malnutrition, and stunted growth in children (Fasano and Catassi, 2005); gynaecological, reproductive and obstetrical dysfunctions (Smecual et al., 1996); skeletal (Blazina et al., 2010), psychiatric, neurological (Collin et al., 1991) and hepatobiliary diseases (Kaukinen et al., 2003); malignomas (Green et al., 2003); increased risk of associated autoimmune diseases (Ventura et al., 2002).
The gluten-free diet is the essential treatment for coeliac patients and requires avoidance of any food or other products containing prolamins from wheat (gliadins), rye (secalins), barley (hordeins) and oats (avenins). However, total avoidance of these prolamins is not so simple because of their presence in unexpected foodstuffs such as meat products, soups, sauces, juices and beers, as well as in food additives like flavorings, emulsifiers, colorings and preservatives that are derived from gluten-containing grains (Gregorek et al., 2006). Some research suggested that some CD patients can tolerate oats, but it also might be contaminated with wheat, barley, and/or rye during harvest, transport, and/or processing (Koerner et al., 2011). Also, industrial starch and other ingredients used in manufacturing pharmaceutical products and food supplements can contain prolamins as impurities.
In the present study, the presence of hidden gluten in a range of dietary products available on the Serbian market has been investigated.

MATERIAL AND METHODS

Thirty five food supplements and thirty samples of dietary foods for infants and young children from Serbian market were analyzed using a commercially ELISA kit (RIDASCREEN® Gliadin, Art. No. R7001) produced by R-Biopharm AG, Darmstadt, Germany. This sandwich ELISA kit has a detection limit of 3 mg/kg of gluten and is based on the monoclonal antibody R5 that reacts with gliadin-fractions from wheat and corresponding prolamins from rye and barley (Valde´s et al., 2003; Mendez et al., 2005). The quantification range of this assay is 5 to 80 mg/kg of gluten. The method has been endorsed by the Codex Committee on Methods of Analysis and Sampling as a type 1 method for determination of the gluten content in gluten-free foods (ALINORM 06/29/23).

Samples extraction and preparation

0.25 g of the homogenized sample of dietary product was weighed into a 10 ml centrifuge tube, a Cocktail-Solution (2.5 ml) was added and the tubes were put on a vortex for 30 s. Samples were incubated for 40 min at 50 ºC, then mixed with 7.5 ml of 80% ethanol and incubated for 1h in a rotary shaker at 45 turns/min. The tubes were centrifuged at 2500 x g for 10 min at room temperature and the supernatants were used for sandwich ELISA analysis. A measured aliquot of the supernatant was removed; diluted 1:12.5 (80 µl + 920 µl) and then 100 µl of this solution were used in the assay.

Gluten determination

Standards and samples were added in duplicate wells on the plate and allowed to incubate for 30 min at room temperature followed by three washing steps. Enzyme conjugate was added to each well and the plate was incubated for 30 min followed by an additional three washing steps. At this point substrate and chromogen were added to each well and allowed to react for 30 min followed by the addition of stop reagent. The absorbance was read at 450 nm on a BioTek EL800 microplate reader (BioTek Instruments, Inc., Winooski, VT) and the data were analysed to determine gluten concentration.

RESULTS AND DISCUSSION

According to the regulations in Serbia (“Sl.glasnik RS”, No.45/2010) the maximum level of gluten in products labelled as gluten-free is 20 mg/kg. In the this study we have determined the content of gluten in thirty five samples of food supplements, produced by different manufacturers, that according to their nature should not contain gluten (Table 1). Among them, only five samples of food supplements had specifically mentioned gluten status. Therefore, these samples are not recognised as gluten-free by CD patients. The obtained results have shown that most of the analyzed food supplements have gluten content less than limit of detection (3 mg/kg) and limit of quantification (5 mg/kg) method. Gluten content of 5-20 mg/kg was determined in three food supplements, and only one analyzed sample had gluten content higher than 80 mg/kg.

Table1. Gluten content in analysed food supplements

Sample	Type of dietary supplement	Producer	Gluten content (mg/kg)
1	multiminerals and vitamins, chewable tablets	A	-
2	multivitamins, granules	B	-
3	multiminerals and vitamins, tablets	B	-
4	multivitamins with fish oil, syrup	C	-
5	vitamin A+D3, drops	D	-
6	**vitamin D3, capsules***	E	-
7	calcium with vitamins A&D, powder	F	-
8	selenium with vitamins, capsules	B	9,8
9	coenzyme Q10 with vitamin and mineral antioxidants, powder	A	-
10	chromium, capsules	B	6,4
11	iron, capsules	B	>80
12	cod liver oil, softgels	G	-
13	fish oil, softgels	H	-
14	**fish oil, softgels***	I	-
15	fish oil, softgels	J	-
16	fish oil, softgels	I	-
17	fish oil, *Ginko biloba* extract+ B vitamins, capsules	K	-
18	fish oil, softgels	A	-
19	glucosamine + chondroitine, tablets	K	-
20	**glucosamine, capsules***	I	-
21	*Aloe vera* gel with glucosamine and chondroitine, drink	A	-
22	royal jelly, powder	L	-
23	royal jelly, powder	M	-
24	honey with propolis and essential oils	N	-
25	propolis and *Echinacea purpurea* extract, drops	O	13.5
26	pollen extract, tablets	P	-
27	whey protein, powder	Q	-
28	**whey protein, powder***	R	-
29	proteins, powder	R	-
30	colostrum, tablets	S	-
31	lactoferrin, capsules	T	-
32	**collagen with vitamin C, tablets***	U	-
33	*Ganoderma lucidum* extract+ *Perna Canaliculus* extract, syrup	V	-
34	chitosan, capsules	F	-
35	*Aloe vera* gel, drink	A	-

Note: "-"= gluten content < 5 mg/kg.
**= labelled gluten status.*

Table 2 shows the results for the dietary foods for infants and young children samples analyzed for the presence of gluten in this study. In four of five of samples of instant baby foods containing only naturally gluten-free ingredients the quantity of gluten have exceeded 20 mg/kg. Considering the high amount of gliadin in these samples, gluten contamination possibly occurred during manufacturing of these products.

Table2. Gluten content in dietary foods for infants and young children

Groups of products	Producer	Number	Gluten content (mg/kg)
baby poridge	A	25	-
instant baby food	B	5	
rice cereal with prebiotics			>80
rice and corn with milk			>80
rice and corn cereals with milk and fruits			>80
cereal with vanilla rich vitamins and minerals			>80
skim milk cereal with rice rich in vitamins and minerals			-

Note: "-"= gluten content < 5 mg/kg

CONCLUSIONS

The most of the naturally gluten-free food supplements analyzed in our study, although not bearing the "gluten-free" label, can be safely consumed by coeliac patients. However, our finding of possible contamination with gluten of the products, claimed to be gluten-free, especially grain-based products, suggested that systematic control of gluten-free dietary products should be obligatory. Thus, the printing of information "gluten-free" on the labels of dietary products exclusively on the basis of ingredients without any laboratory analyses should not be allowed.

ACKNOWLEDGEMENTS

This research was part of the Project No 46009 "Improvement and development of hygienic and technological procedures in producing food animal origin in order to obtain high-quality and safe products competitive on world markets", funded by the Ministry of Education and Science, Republic of Serbia.

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ENRICHMENT OF GLUTEN-FREE EXTRUDATES WITH FLAXSEED

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ABSTRACT: This study aimed to investigate the influence of flaxseed addition on technological and nutritional quality of gluten-free extruded product. Ground flaxseed was added in amounts of 3, 5 and 7 g/100 g to corn meal and rice flour mixture (1:1) and extruded by twin-screw co-rotating extruder. Enrichment with flaxseed resulted in significantly lower expansion ratio and higher bulk density of extrudates, but peak viscosity, water absorption and water solubility indices were barely modified. The content of protein, total fibre, and unsaturated, saturated and omega-3 fats significantly positively correlated to the flaxseed amount. In all extrudates saturated fats were lower than 1 g/100 g, and the average energy value was 379±3 kcal/100 g. In 7% flaxseed enriched sample, the total phenolics content determined by Folin-Ciocalteu method was higher by 45% compared to the non-enriched, and its lignan content determined by GC-ECD method was 14.44 mg/100 g vs. 0.06 mg/100 g in the control sample. However, the antioxidant capacity evaluated using DPPH and ABTS assay was similar among samples. In conclusion, the addition of ground flaxseed in amount of 5% can be recommended for the enrichment of gluten-free snack or breakfast cereal. Such product could be labelled as a source of fibre and high in omega-3 fatty acids according to a valid EU regulation.
Key words: *antioxidants, extrusion, gluten-free, flaxseed, lignans, omega-3 fatty acids*

INTRODUCTION

Gluten-free cereal products are known to be of low nutritive value compared to the standard products that they are intended to replace. They are mostly produced from rice and corn because of their hypoallergenicity. At the same time, these products can be high in calories and fat that has been added in order to enhance overall acceptability (Niewinski, 2008).
Food market offers broad spectrum of snacks and breakfast cereals produced by different processing techniques. One of the most popular technique is extrusion cooking. Snack products are extruded at low moisture content, high temperature and high shear process that is characterised by forming a melt from the starchy ingredient. This leads to a more expanded product that melts in the mouth. Starch is the main constituent of the extruded snacks and is responsible for most of their structural attributes. Thus, extruded products are dense in energy but are nutritionally poor. However, there are many possibilities to incorporate beneficial nutrients into them (Dehghan-Shoar et al., 2010).
Functional ingredient which is often added to cereal-based products is flaxseed. Flaxseed is the leading plant food source of the ω-3 fatty acid, phytoestrogen compounds known as lignans, and soluble polysaccharides. In comparison to cereals, flaxseed is 200-fold higher in lignans and 3 times higher in total phenolics (Čukelj et al., 2011b). Incorporation of flaxseed in the diet is particularly attractive for the development of foods with specific health advantages (Oomah, 2001). For instance, ω-3 fatty acids show great promise in prevention of cardiovascular diseases (Lavie et al., 2009), lignans are associated with a reduced breast cancer risk (Cotterchio et al., 2007), and polyphenols are known antioxidants (El Gharras, 2009).
The aim of this study was to investigate the influence of flaxseed addition on technological and nutritional quality of gluten-free extruded product manufactured from corn meal and rice flour. The flaxseed was added at three levels. Physical and chemical properties, total phenolics and lignan content, as well as antioxidant capacity of flaxseed enriched gluten-free snack were examined.

MATERIALS AND METHODS

Extrusion cooking

Extrudates were produced from corn meal (Naše Klasje, Croatia), rice flour (Davert Muhle, Germany), and flaxseed (Suban, Croatia). Flaxseed was milled to a 1 mm particle size. Equal amounts of corn meal and rice flour were mixed with flaxseed at 3, 5, or 7 kg/100 kg of flour blend. A control sample without flaxseed was prepared in the same way. Blends were extruded in twin-screw co-rotating extruder APV SB 65 (Baker Perkins, UK) consisting of two independent zones of controlled temperature in the barrel set at 130°C and 150°. The average screw speed was 300 rpm. The feeding rate was 312.5 kg/h, water was injected directly at 32.9 L/h, and lecithin was used for lubrication at 1.4 kg/h. The product coming out from the extruder through a circular die of 3.3 mm diameter was cut into short pieces. Extrudates were dried in a forced ventilation air tunnel that was set at 90°C in the first and at 30°C in the second section.

Physical properties of extrudates

Expansion ratio was calculated as the ratio between the average diameter of 30 extrudate samples, measured with calliper, and the die diameter. For the determination of bulk density, volume of 100 g of extrudates was measured in five replicates and the average ratio of mass to volume was calculated. Water absorption index (WAI) and water solubility index (WSI) were determined in duplicates using the method of Anderson et al. (1969). Pasting properties of the milled extrudate (10 g, with correction to 14% of moisture) dispersed in 105 ml of deionized water were determined twice according to AACC Method 22-08.01 (1999) with a MicroViscoAmylo-Graph (Brabender, Germany).

Chemical composition of extrudates

Chemicals of adequate analytical purity were purchased from the local market. Standards of lignans were a kind gift from Oy Separation Research Ab (Turku, Finland). Moisture, protein and ash were determined according to AACC methods (1999). Total dietary fibre was determined following an AOAC 2009.01 method (2011) using Megazyme kit. Fat content of the sample was measured using a Soxhlet extraction, after an acid hydrolysis. Fatty acid composition was determined according to ISO Methods 5509:2000 and 5508:1990. Gas chromatography of fatty acids was performed using an Agilent Technologies 6890N Network GC System equipped with a FID detector and a 60m x 0,25mm capillary column DB-23 (Agilent, USA). Helium was carrier gas at a flow rate of 1.5 ml/min. Sample was injected with a splitting system 1:30. Injector was set at 250°C and detector at 280°C. The column was heated from 60 to 220°C, at a 7°C/min rate. Available carbohydrates and energy value of extrudates were calculated according to Joint FAO/WHO Report (1998).

Prior to determination of phenolic compounds, lignans and antioxidant activity, extrudates were defatted. Total phenolic content (TPC) and antioxidant activity were determined spectrophotometrically (UNICAM Heλios β, England). The extracts of phenolics were prepared as described by Li et al. (2009), but adjusted for quantities using 10-times bigger amounts. TPC was measured according to Yu et al. (2002), with modifications (Čukelj et al. 2011b). The results were expressed as mg of ferulic acid equivalents (FAE) per 100 g of dry sample. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging capacity of extracts was assessed by the method of Zhou and Yu (2004) with following modifications: to 1.9 ml of 0.06 mM DPPH in methanol, 100 µL of extract was added, and the free radical scavenging capacity was evaluated by measuring the decrease of absorbance at 517 nm. Antioxidant capacity was expressed as mmol/L Trolox equivalents. The Trolox equivalent antioxidant capacity of extrudate extracts was as well estimated by the ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) radical cation decolorization assay (Re et al., 1999). Extraction and quantification of lignans in extrudates were done on the same GC as fatty acids but connected to electron-capture detector as described earlier by Čukelj et al. (2011a).

All chemical analyses were the means expressed with standard deviation (SD) of two replicates except total phenolics and antioxidant activity tests that were conducted in triplicates.

RESULTS AND DISCUSSION

Physical properties of extrudates

In this study, we investigated the effect of incorporating ground flaxseed on the nutritional and physical properties of gluten-free extruded product. When developing novel nutritious food, it is necessary to monitor its physical properties as they greatly affect consumer acceptability. Product with the added flaxseed were of lower expansion ratio (ER) and higher bulk density (BD) than non-enriched sample but the difference was statistically significant only at $p \leq 0.1$ (Table 1). BD is an index of the extent of puffing, thus as the volume of extruded products increases the BD decreases. Cheng and Friis (2010) showed that the maximum expansion is obtained with pure starches, while dietary fibre and protein have a negative effect on the expansion. Flaxseed contains considerable amount of both, so negative expansion was expected. Similar trend for the ER and BD was obtained with extruded corn snack enriched with flaxseed flour at 5-20% (Ahmed, 1999). To compensate and produce well expanded product, extrusion processing at high temperature and shear should be tested.

The amount of degraded starch granules is positively related to WSI and inversely to WAI. Flaxseed addition slightly affected WAI and WSI of extrudates (Table 1) and the differences between samples were statistically significant only at level $p \leq 0.1$. Solely the control was different from all the other samples in WAI at $p=0.05$, and in WSI it differed from the sample with 7 % of flaxseed at $p=0.08$. The similar results were obtained by analysing pasting properties. The enriched samples begun to gelatinise earlier at lower temperature, but the peak paste viscosity and the temperature thereof did not significantly differ (Table 1). The viscosity of a paste depends to a large extent on the degree of gelatinisation of the starch granules and the extent of their molecular breakdown (E-Dash et al., 1983). Similar viscosity of extrudates obtained after enrichment could be due to the fact that soluble fibre from flaxseed can enhance starch gelatinisation thus compensate for starch dilution effect.

Table 1. Effect of added flaxseed on the physical properties of the extrudates (average ± SD)

Flaxseed addition (%)	Expansion ratio	Bulk density (g/L)	WAI (g/g)	WSI (g/g)	Peak viscosity (BU)
0	4.60±0.28	59.2±0.2	11.07±0.03	0.27±0.03	38.5±3.5
3	4.33±0.26	60.7±0.2	11.73±0.21	0.24±0.03	51.0±2.8
5	4.08±0.22	62.6±0.2	11.87±0.36	0.23±0.01	45.0±0.7
7	3.66±0.26	86.7±0.4	12.10±0.40	0.21±0.01	42.5±4.1

Chemical characteristics of extrudates

As the proportion of flaxseed in the extrudates was higher, protein, fat, and dietary fiber also reflected a significant growth (Table 2). In the same time, energy value of extrudates did not significantly change and the content of saturated fatty acids remained below 1 g/100 g. The dominant were unsaturated fatty acids: linoleic, oleic and linolenic. By adding flaxseed, the content of α-linolenic acid progressively enhanced, and consequently the ratio of omega-6 to omega-3 fatty acids was almost 1:1. The recommended daily intake of omega-3 fats (250 mg) could be met by consuming 40, 31 or 24 grams of extrudates enriched with 3, 5 or 7 % of flaxseed, respectively. Extrudates with added flaxseed at 5 and 7 % could be labelled as a source of fibre and high in omega-3 fatty acids according to European Regulation 1924/2006 on nutrition and health claims made on foods.

Table 2. Chemical characteristics and energy value of 100 g of extruded products (average±SD)

Parameter	Flaxseed addition level (%)			
	Control (0)	3	5	7
Moisture (g)	7.84±0.01	7.75±0.04	7.81±0.06	7.89±0.21
Ash (g)	0.55±0.01	0.61±0.03	0.63±0.01	0.63±0.02
Protein (g)	7.67±0.02	8.04±0.01	8.19±0.03	8.29±0.04
Fat (g)	2.66±0.01	3.38±0.15	3.95±0.35	4.70±0.05
of which saturated	0.52±0.03	0.65±0.01	0.85±0.10	0.90±0.01
of which unsaturated	2.04±0.05	2.63±0.02	2.96±0.15	3.69±0.01
Omega-3	0.34±0.04	0.65±0.01	0.73±0.09	1.10±0.01
Available carbohydrates (g)	79.03	77.71	76.41	75.09
Fibre (g)	2.25	2.51	3.01	3.40
Energy (kcal/kJ)	375/1566	378/1579	380/1587	383/1600
Antioxidant capacity (mM Trolox/100 g)				
DPPH	397.3±6.3	385.8±1.5	354.5±17.1	329.0±7.6
ABTS	347.9±34.8	349.1±36.3	360.4±17.1	341.8±16.3

In products enriched with flaxseed, the TPC and total lignan content were significantly higher (at $p<0.001$) compared to the control sample (Fig. 2). In the sample with 7% of added flaxseed, TPC was almost two times higher compared to the control sample. In a previous study, TPC in flaxseed was around 3 times higher compared to cereals (Čukelj et al., 2011b), thus the higher level of TPC in extrudates was expected. We applied alkaline hydrolysis prior to extracting phenolics in order to include both free and bound phenolics. It has been proven that bound phenolics make more than 74% of all the phenolics in rice, and more than 69% in corn (Adom and Liu, 2002). The similar growing trend with flaxseed addition was observed for total lignans, and both TPC and lignan content significantly correlated ($r=0.986$, $p<0.05$). In enriched extrudates the main lignan contributing to more than 97% of total lignan content was secoisolariciresinol, while lariciresinol and pinoresinol were also detected. Cotterchio et al. (2007) showed that among Western women, lignans contribute approximately 70% of total phytoestrogen intake, i.e, nearly 2 mg/day. Beneficial effect on menopausal health was observed with the intake of 30 mg/day of soy isoflavones (Kurzer, 2003), thus the intake of extrudates enriched with flaxseed could drastically improve the amount of phytoestrogen in everyday diet and thus, health status.

Antioxidant capacity of extrudates was double examined ABTS and DPPH methods, and in both measurements we compared it with the water soluble vitamin E analogue - trolox (Table 2). The results obtained with both methods showed to be similar. Although the flaxseed enriched extrudates were higher in TPC and lignan content, there was no significant difference in antioxidant capacity between the samples. The DPPH values show even slight decrease in antioxidant capacity of extrudates with more added flaxseed although the phenolics are considered to be one of the main carriers of the antioxidant capacity (Brennan, 2011). We assume that during the one month storage of extrudates at room temperature, before DPPH and ABTS tests were conducted, the oxidation of unsaturated fats occurred. In turn this resulted in a similar antioxidant activity of control and enriched samples, since enriched samples were higher in fats. Not only phenolics, but also other components of flaxseed such as tocopherol and omega-3 fatty acids act as antioxidants. Our study indicates that the lignans and other phenolics are not the most sensitive flaxseed antioxidants quickly used up during processing and storage. This is in agreement with the study by Liukkonen et al. (2002), which showed that rye lignans and alkaline-extractable phenolic compounds remain almost unchanged during baking. On the other hand, tocopherols and tocotrienols showed a decrease in the amount, during sourdough baking process. Further studies should investigate the effect of storage time on the oxidative stability of extrudates enriched with ground flaxseed.

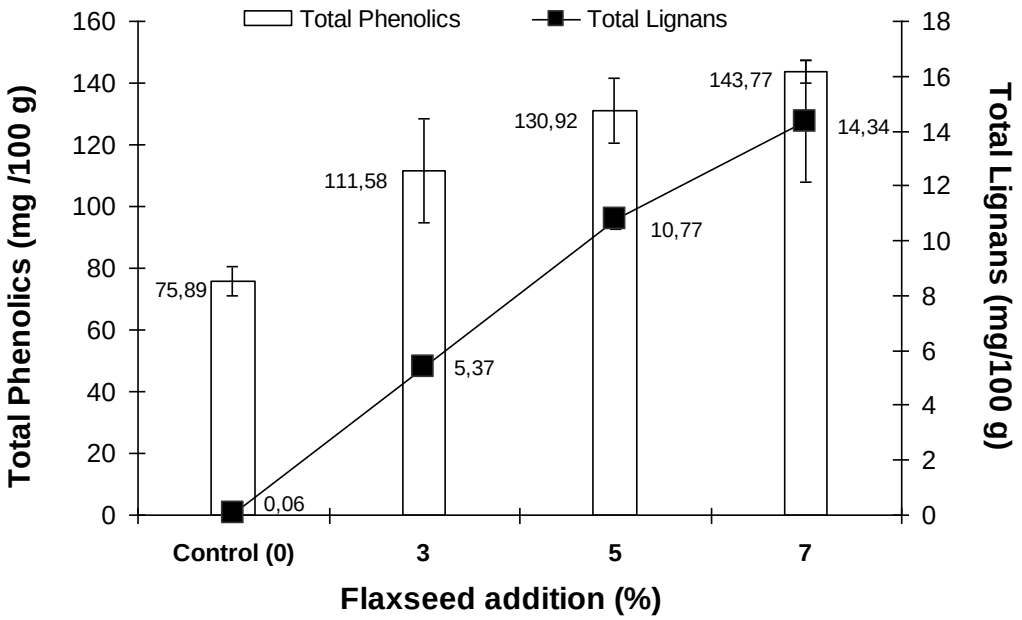

Figure 1. Total phenolic content (TPC; mg FAE/100 g±SD) and total lignan content (mg/100 g±SD) of extruded products (dry basis).

CONCLUSIONS

The proximate composition reflects better nutritive profile of the enriched extrudates in terms of fibre amount, protein content, and fatty acid composition, in comparison to non-enriched. In particular, the content of omega-3 fats, phenolic compounds and lignans improved upon enrichment with flaxseed. However, some physical characteristics of extrudates, especially expansion ratio, were negatively affected by flaxseed addition. Taking into account both physical and nutritive characteristics of the extrudates, 5% of flaxseed could be incorporated in a gluten-free snack or breakfast cereals, depending on the formulation and process conditions. Such information could be helpful to food producers in development of extruded material as a novel product for various consumers demanding gluten-free, nutritionally enhanced or convenience foods.

ACKNOWLEDGEMENTS

We thank the staff of Naše Klasje, Ltd. Zagreb, especially Zoran Šimunić and Matija Ivandić, for their valuable technical help in extrusion process.

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HEALTHY BREAD AS AN ALTERNATIVE TO TRADITIONAL BREAD

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ABSTRACT: Today, bread is part of the culture and eating habits of most people and can be considered one of the most consumed foods in the world. The bread is made from a simple mixture of flour, water, salt and yeast, and it is an extremely versatile food and with a very good digestibility. Since it was first produced for thousands of years, it has undergone changes both in terms of production methods and in terms of its constitution, thus giving place nowadays to a wide variety of breads available on the market. The objectives of this study were to compare the sensory and nutritionally attributes of a special healthier bread, "Pão São", which is a recent product in the Portuguese market. Nutritionally this type of bread turned out to be a food with high nutritional value, with a low salt content (0.4%, about half of traditional bread), and being a good source of omega-3 (0.67%), fibre and protein when compared with the traditional bread. These differences are mainly due to the wide variety of raw materials selected to the preparation of this bread, including the mixture of flour (wheat flour type 150 and 65 and rye flour type 70), oatmeal, sunflower and linseed seeds, soy, fish oil extracts rich in omega 3, malt and milk protein. From the results of the sensory analysis, conducted by a panel of 40 untrained tasters aged 7 to 63 years, it was found that the traditional breads from the region closest to the marketing of healthy bread were preferred by the tasters, especially for their wood and bread flavour, as well as elasticity. The healthy bread was evaluated as a denser bread, with a more intense fermented flavour.
With this work it was concluded that the healthy bread is a nutritious and healthy bread, being currently recommended by the Portuguese Foundation of Cardiology. However from the sensory point of view, consumers still slighly prefer the traditional bread.
Key words: *bread, density, texture, colour, salt, sensorial analysis*

INTRODUCTION

Bread is consumed since prehistoric times, and may have been the first food produced by humans. The advantages of a diet rich in fibres are well known, and in present times the concern about the low level of fibre intake in most diets is growing. Among the ingredients used to produce bread, flour is undoubtedly the most important. Wheat or rye flours are an excellent source of fibre, particularly insoluble fibre (Leon and Rosell, 2007), thus having positive effects on human health, such as decreasing the risk of coronary heart disease, hypercholesterolemia, obesity and diabetes (Leon and Rosell, 2007). Besides, wheat is also a major source of antioxidants like phenolic acids or flavonoids (Pathlrana and Shahidi, 2007).
According to Brandt *et al*. (2005), the amount and type of the ingredients of the dough affect its properties; among these are, besides flour, the water, fat or other additives. Also the performance of accurate methods of blending, kneading and fermentation (temperature, intensity, duration, etc.) have a marked influence on the dough quality. The bread presents better organoleptic characteristics when it is cooked from fresh flour. However, when techniques such as storage, distribution and sale of fermented, partially cooked, frozen or refrigerated dough, allows the baking of bread just before it is purchased or consumed, the bread still has a good flavour despite the aroma being slightly compromised (Brandt et al., 2005).
According Hoseney (1991) the bread quality involves the following factors: the gas concentration, elasticity, resistance of the dough and capacity of gas retention. The

prolonged fermentation with artificial yeast, such as lactic acid bacteria, can control the action of bacteria and improve the availability of nutrients (Brandt et al., 2005). The quality of the bread can be evaluated by physical, physico-chemical (macroscopic or microscopic), microbiological and sensory properties (Hoseney, 1991).
In Portugal bread is a very consumed food, presented to the consumer in many different varieties, according to the main cereals used (corn, wheat, rye, mixtures, whole flours), as well as the manufacturing process and type of fermentation (Almeida et al., 2008).

MATERIAL AND METHODS

Samples

Two different types of healthy bread were bought from the manufacturer “Fabrica do Pão” located in Seia, Portugal, and for each type three samples were taken for analysis. The healthy bread samples differed from one another because one contained a different ingredient, being this lupine flour. Also Regional bread samples (seven samples) from Viseu region, in Portugal, were analyzed for comparison.

Chemical analyses

The analysis to the chemical properties done were: moisture content, water activity, ash content, crude fat, crude fiber content, protein content, all done following the official methods of AOAC (2000). The analysis of the salt content was done by the Mohr Method, being the determination of carbohydrates calculated by difference.

Physical analyses

The physical properties evaluated in this study were: size, color, density, alveolar characterization and texture. The dimensions and volume of the loaves were measured in terms of thickness, width and length. The volume was calculated by approximating the form to an ellipsoid.
In this study, the color parameters were evaluated using a colorimeter chroma meter (Minolta, Japan) expressing the results in the CIELab system coordinates: L* which is the brightness and varies between 0 (black) to 100 (white), the a* ranges from -60 (green) to +60 (red) and b* ranging between -60 (blue) to +60 (yellow).
The alveolar characterization was done by image analysis, with the program "Image J". From each sample, 5 fresh slices were prepared with a thickness of 10 mm (pattern cutting).
Textural analysis was made by Texture Profile Analysis with a texturometer TA-XT2 from "Stable Microsystems".

Sensorial analyses

Sensory analysis was performed in a laboratory prepared for that purpose, on the day of delivery of the samples by a panel of 40 untrained tasters, aged between 7 and 63 years, who were asked to rate the following attributes: crumb colour, crust colour, aroma (bread, firewood and fermented), taste (bread, wood or fermentation), elasticity, density, and finally the overall appreciation. In this test the taster expressed the intensity of each attribute through a scale where verbal Hedonic expressions are translated into numeric values in order to allow statistical analysis. The scale of values varied from 0 (less intense) to 10 (more intense).

RESULTS AND DISCUSSION

After analyzing the different types of bread separately, traditional bread and healthy bread, it was intended to compare these two types of bread in terms of the different properties evaluated, chemical, physical and sensorial. The results presented are the mean, and standard deviation, of the values found for different samples of traditional bread (N = 7) or healthy bread (N = 2).

Chemical properties

Figure 1 shows the means values, with standard deviations, of the chemical properties of the two types of bread studied. From the graphs it is observed that the traditional samples have, in average, higher moisture, carbohydrates, ash and salt contents, whereas the healthy bread samples show higher protein, fat and fibre contents. Some of these characteristics can be explained by the flours used in the manufacture of the healthy breads, which are richer in essential fatty acids (so called good fats), proteins and fibres.

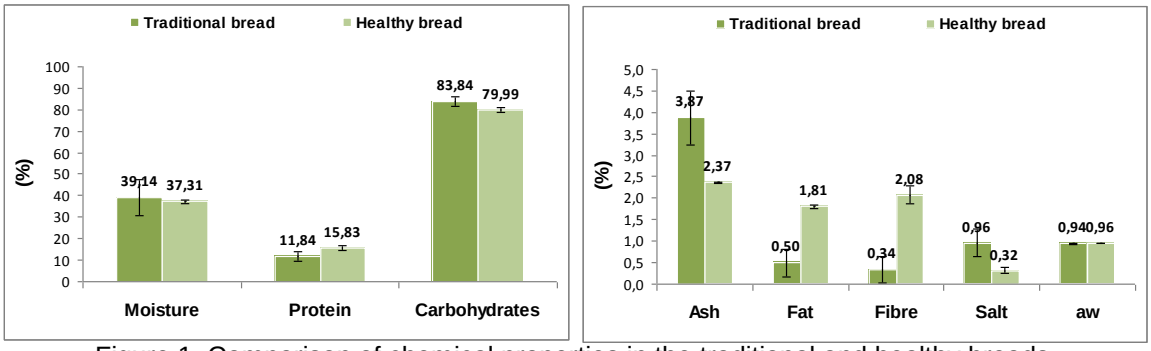

Figure 1. Comparison of chemical properties in the traditional and healthy breads.

Physical properties

From the analysis of the graphs in Figure 2, it is evident that both types of bread do not differ substantially in terms of colour. The healthy bread presents itself darker, with lower L, either in the crust or in the crumb, which is a result of the type of flour used. Regarding a* this parameter is also higher in the healthy bread (crust and crumb), which means that the red coloration is stronger in this case. As to b*, its value is also higher in the healthy bread crumb, being slightly lower in the crust. These results indicate that the healthy bread is browner than the traditional bread.

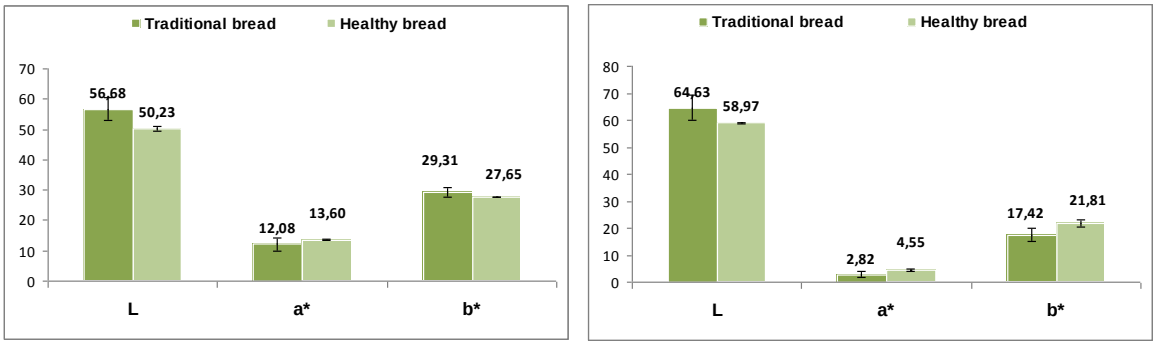

Figure 2. Colour parameters in the traditional and healthy breads. Left: crust, right: crumb.

Table 1 presents the results obtained for the physical properties examined, including alveolar characterization, for both groups of samples, traditional and healthy breads. It can be seen that, in general, the healthy samples showed higher volume and increased porosity, when compared to the traditional samples.

Table 1. Physical properties of the bread samples analyzed.

Type of bread	Whole bread volume (cm^3)	Slice alveolar characterization			
		Area ($pixel^2$)	Nº Alveolus	% Alveolar	Size ($pixel^2$)
Traditional	647,69±135,56	1627995,86±926590,85	241,23±90,75	22,93±11,34	7102,86±3901,10
Healthy	671,22±68,09	2226055,90±220649,70	254,30±9,19	25,70±1,98	8689,90±505,02

Results are given as mean ± standard deviation.

From the analysis of Figure 3, which shows the textural properties of both types of bread studied, it is found that traditional bread show higher elasticity, higher values of springiness, while being softer, lower values of hardness and chewiness. As regards cohesiveness, this is very similar for both types of bread analysed.

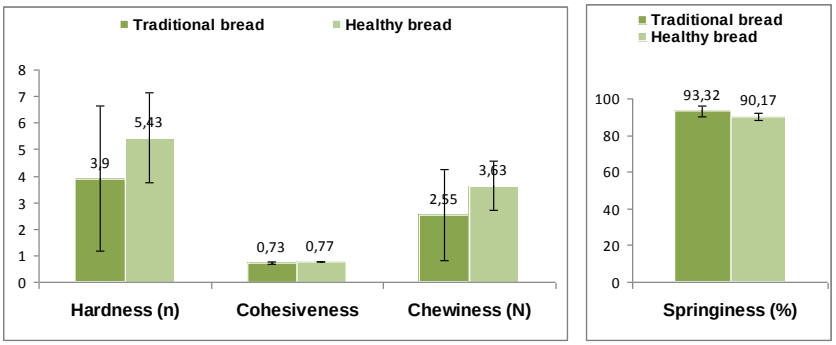

Figure 3. Textural properties in the traditional and healthy breads.

Sensorial properties

The graphs in Figure 4 show the results of sensory evaluations made for the two kinds of bread. Based on the results, it appears that in sensorial terms, and with regard to aroma (bread, wood, fermented), the samples of traditional bread are not distinguishable from those of the healthy bread. However, with respect to colour, either in the crust or the crumb, the healthy bread was identified as having a more intense coloration, which is in accordance with the results obtained when measuring colour. Also the healthy bread was perceived as being less tasty in terms of bread or wood taste, less elastic, more dense and finally in terms of global appreciation it was valued less than the traditional bread. Still the differences were not so important, so that in general terms both breads were considered equally acceptable.

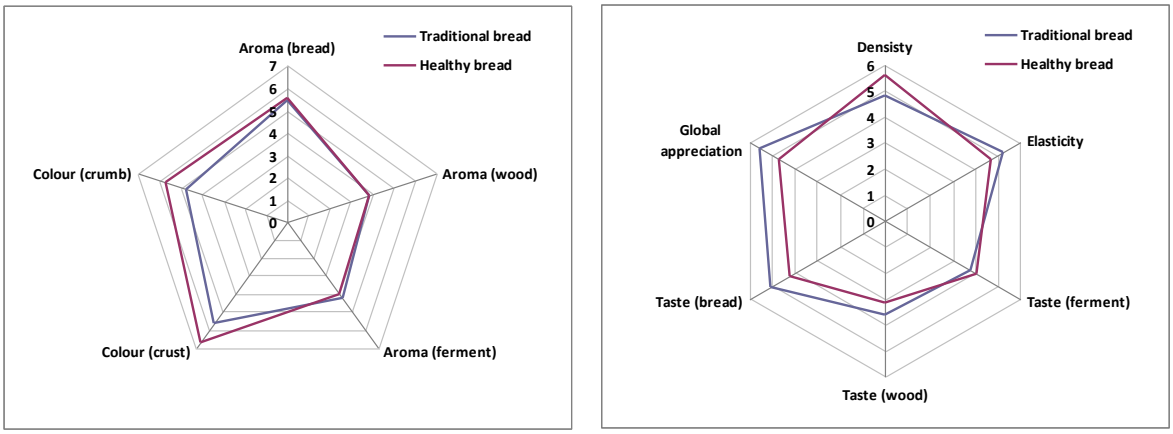

Figure 4. Sensorial evaluation of the traditional and healthy breads.

CONCLUSIONS

The results from this work showed that both types of bread were different in terms of the different properties analyzed. In nutritional terms, the healthy bread showed higher protein content, being also much richer in terms of fibre and fat (due to the presence of important essential fatty acids). Also this type of bread had a much lower salt content, with important health benefits. In terms of physical properties, the healthy bread is browner and with increased porosity. Furthermore, this type of bread is harder when compared to the traditional bread. In sensorial terms, both breads were evaluated in a proximal way, noticing however, that the traditional bread was slightly preferred.

ACKNOWLEDGEMENTS

The authors thank the financial support to the research centre CI&DETS (IPV - Viseu).

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INFLUENCE OF *ORIGANUM HERACLEOTICUM* L. ESSENTIAL OIL ON REDUCTION OF *STAPHYLOCOCCUS AUREUS* USING BROTH MODEL MEDIA

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ABSTRACT: *Staphylococcus aureus* is considered the third most important cause of disease in the world among the reported foodborne illnesses. Pathogenesis of *S. aureus* depends on the ability of the strain to survive, multiply under a variety of conditions and produce various extracellular compounds. This study was undertaken in order to investigate the effectiveness of the *Origanum heracleoticum* L. essential oil on growth inhibition and survival of *S. aureus* isolated from food using broth model media and ability of essential oil to reduce coagulase activity. Cell viability assays were carried out with exposure of *S. aureus* to various concentration of the *O. heracleoticum* L. essential oil (1.0, 0.5, 0.3, and 0.1 µL/mL) in time intervals 15, 40, 60, 120 and 240 min.
The results showed that *O. heracleoticum* L. essential oil after only 15 min at 1 µL/mL completely inhibited the growth of *S. aureus.* Lower concentrations of essential oil (0.5, 0.3 and 0.1 µL/mL) inhibited the cell viability of *S. aureus.* After 1.5h, 1 and 0.5 µL/mL of essential oil suppressed physiological attributes of the tested *S. aureus* strains - coagulase activity. After 24h, tested essential oil at 0.3 µL/mL suppressed coagulase activity.
The oil interfered on the coagulase activity in a dose-dependent manner. *O. heracleoticum* essential oil could be a novel antimicrobial with capability to suppress some physiological characteristics, in addition to inhibit the growth and survival of pathogen bacteria in foods, particularly *S. aureus.*
Key words: *Staphylococcus aureus, essential oil, Origanum heracleoticum* L.

INTRODUCTION

Staphylococcus aureus is considered the third most important cause of disease in the world among the reported foodborne illnesses (Boerema et al., 2006). For intoxication caused by *S. aureus* the pathogenesis depends on the ability of the strain to survive, multiply under a variety of conditions and produce various extracellular compounds (Shale et al., 2005). Haemolysins, nuclease, coagulase, lipase and staphylococcal enterotoxins are among the extracellular toxins and enzymes produced by *S. aureus.* As staphylococcal enterotoxins are heat stable, they may be present in food when *S. aureus* is absent (Balaban & Rasooly, 2000).
The growth of *S. aureus* in food presents a potential public health hazard because many strains produce one or most enterotoxins (SEs) that cause food poisoning if ingested (Akineden et al., 2008). Moreover, the ability of this microorganism of acquiring resistance to practically all useful antimicrobials is a cause of great concern for public health (Gibbons, 2004). Staphylococcal food poisoning frequently involves foods that require considerable handling during preparation and that are kept at slightly elevated temperatures after preparation (Le Loir et al., 2003).
Consumers have demanded more natural foods, with low levels of chemical preservatives and less processed, however still possessing a long shelf-life. Also, food legislation has restricted the use of some synthetic antimicrobials based on a possible toxicity for consumers (Burt, 2004). In this panorama, aromatic plants have emerged as effective compounds to provide microbiological safety of foods.
Aromatic plants are rich in essential oils, which are composed of many compounds (carvacrol, thymol, eugenol, cinnamic acid, citral, pinene,) characterized by a prominent

antimicrobial activity. This trend prompts a particular increased interest in the use of essential oils as antimicrobial compounds to be applied in food conservation. Among the aromatic plant from family *Lamiaceae* (*Labiateae*), genus *Origanum* has a special position. This is basically due to their major components carvacrol and thymol, having important antioxidant and antimicrobial properties (Ozkan et al., 2003). Carvacrol and thymol, the two main phenols that constitute about 78–85 % of oregano oil, are mainly responsible for the antimicrobial activity of the oil (Kokkini et al., 1997). It should be mentioned that the biological activity of essential oils depends on their chemical composition, which is determined by genotype and influenced by environmental and agronomic conditions.
This study was undertaken in order to investigate the effectiveness of the *Origanum heracleoticum* L. essential oil in inhibiting the growth and survival of *S. aureus* isolated from foods using broth model media.

MATERIAL AND METHODS

Plant Material

Aerial parts of *O. heracleoticum* L. were collected during blooming stage (August 2009) from the locality Kamendol near Smederevo, Serbia. The plant material was dried under laboratory conditions (20-25 ºC). Institute of Medicinal Plant Research Dr. Josif Pančić identified the plants and voucher specimens were stored in the herbarium of the Institute of Medicinal Plant Research Dr. Josif Pančić.

Isolation of the essential oil

The essential oil was isolated from dried plant material by hydro-distillation according to the standard procedure reported in the Sixth European Pharmacopeia (2008). Distillation was performed using Clevenger type apparatus, for 2.5 hours. The resulting essential oil was dried over anhydrous sodium sulfate and stored at 4 ºC.

Bacterial strain

S. aureus obtained from the Microorganism Collection, Laboratory of Food Microbiology, Institute of Food Technology, Novi Sad was used as test strain in all experiments. This strain was isolated from goat whey sample by the standard procedures according to ISO 6888-1 (2003). Stock culture was kept on Nutrient Agar (NA) slants under refrigeration (7 °C ± 1 °C). Inocula used in the assays were obtained from overnight cultures grown on NA slant at 37 °C. A loopfull of the culture was diluted in sterile saline solution (0.85 g/100 mL) to have a final concentration of approximately 10^8 colony forming unity per mL (cfu/mL) adjusted according to the turbidity of 0.5 McFarland standard tube. Final concentration of the inoculum in the medium used for the antimicrobial assays was approximately 10^6 cfu/mL.

Cell viability assay

Cell viability assays were carried out using the viable cell count method. For this aliquots (100 µL) were taken at 15, 40, 60, 120 and 240 min, serially diluted in sterile peptone water (0.1 g/100 mL) and spread-plated onto sterile Baird–Parker agar. After 24 h of incubation at 37 °C, colonies were counted and the results were expressed in log of cfu/mL. Control flasks without essential oil were tested in the same way.

Effect of oil on the activity of coagulase

After 1.5 and 24 h, 100 µL of culture grown on Brain Heart Infusion Broth (BHI) with addition of essential oil (1.0, 0.5, 0.3 and 0.1 µL/mL) was used to test the activity of coagulase. After the incubation period, the activity of coagulase was estimated by the standard procedures according to ISO 6888-1 (2003), and the results were expressed in level (-; + to +++) of the coagulase test regarding the size of the formed plasma clot. All antimicrobial assays were carried out in duplicate and the results were expressed as average of the parallel assays.

RESULTS AND DISCUSSION

From the collected plant material of *O. heracleoticum* L. total of 2.05 % (v/w) of essential oil has been isolated by the process of hydro-distillation. In our previous investigation (Cabarkapa et al., 2011) twenty six components (92.86 %) were identified as constituents of this essential oil by GC/MS analysis. The major components were carvacrol (69.00 %), p-cymene (10.50 %), thymol (7.94 %) and γ-terpinene (2.86 %). Except β–caryophyllene (1.53 %) and β–bisabolene (1.01 %) the amount of all remaining oil components was less than 1 %. In our previous experiments 0.2 and 0.78 µL/mL were found as minimum inhibitory concentration and minimum bactericidal concentration, respectively, of *O. heracleoticum* L. essential oil toward the assayed strains of *S. aureus* (not published data).
Obtained results of the effect of different concentrations of essential oil (0.5, 0.3 and 0.1 µL/mL) on the cell viability (kill time) of *S. aureus* are shown in Figure 1. Results showed that the growth of *S. aureus* was completely inhibited after only 15 min of exposure to essential oil at concentration 1 µL/mL (this result is not shown in Figure 1 because of its simplicity). After 15 min of exposure to essential oil at concentration 0.5 µL/mL initial cell count was decreased for 1.46 log cfu/mL and after 40 min total elimination of the microbial initial inoculum was evaluated. According to Barros et al. (2009) a compound is considered as having a strong cidal effect when it is able to cause a decrease of 99.9% (3 log cycles) of the initial inoculums. Therefore, strong cidal effect (total elimination of the microbial initial inoculum) was found after 15 min of exposure to the oil at 1 µL/mL and after 40 min of exposure to the oil at 0.5 µL/mL.

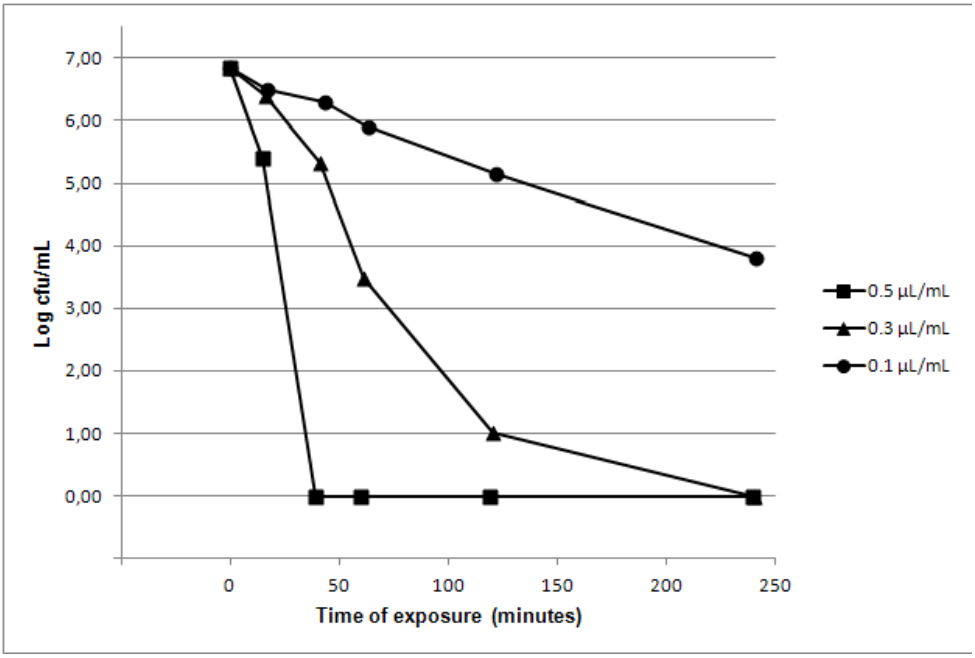

Figure 1. Effect of different concentrations of *O. heracleoticum* L. essential on the cell viability of *S. aureus*

After 15 min of exposure to concentration 0.3 µL/mL, tested essential oil provided a static effect along the evaluated interval (reduction of microbial initial inoculum was ≤0.4 log). The lowest tested concentration of oil (0.1 µL/mL) after 15 and 40 min provided a static effect along the evaluated interval (reduction of microbial initial inoculum was 0.35 and 0.54 log, respectively). Cidal effect was found after 60 min of exposure to the oil at 0.3 µL/mL (reduction was 3.38 log) and after 240 min of exposure to the oil at 0.1 µL/mL (reduction was 3.04 log).
Barros et al. (2009) found that the treatment of *S. aureus* with essential oil of origanum with 54 % of carvacrol had cidal effect after 15 min of exposure to the oil at 1.2 µL/mL. After 120

min, the oil at 1.2 µL/mL decreased the cell count to <2 log cfu/mL. In the same investigation tested essential oil at concentration 0.3 and 0.6 µL/mL provided a static effect along the evaluated intervals. The oil at 0.6 and 1.2 µL/mL caused a significant decrease ($P < 0.05$) in the bacterial count in comparison to the control assay. Otherwise, it was not found at 0.3 µL/mL.

Origanum heracleoticum essential oil is rich in phenolic compounds, which are believed to be responsible for its prominent antimicrobial activity. The major antibacterial components of these oils are carvacrol and its isomer thymol. The precise target(s) of the antibacterial action of essential oils and their components has not yet been fully established. Carvacrol and thymol are capable of damage the outer membrane of bacteria and increase the general permeability of the cytoplasmic membrane, leading to leakage of ATP (Burt 2004). Cytoplasm membrane disturbances, rupture of proton motive force and cytoplasm content coagulation are some mechanisms involved in the antimicrobial properties of essential oils. These findings show an interesting inhibitory effect of *Origanum heracleoticum* essential oil toward the cell viability of *S. aureus*, a fast and steady bacterial kill rate. Kill-time curve also showed a clear relationship among the extent of inhibition and the oil concentration and time of exposure.

The effect of *O. heracleoticum* essential oil on the coagulase activity of *S. aureus* cells is shown in Table 1. The oil at 1, 0.5 and 0.3 µL/mL caused an inhibition of coagulase activity after 90 min (provided large reduction in coagulase activity, where no clot was formed after incubation of the cultures in the presence of rabbit plasma). At 0.1 µL/mL the coagulase activity found for the cells were in a range of +++ (level 3) after 90 min to ++ (level 2) after 24 h.

Table 1. Effect of *O. heracleoticum* L. essential on coagulase activity in *S. aureus* cells

Treatment of essential oil	90 min	24h
1 µL/mL	- - -	- - -
0.5 µL/mL	- - -	- - -
0.3 µL/mL	- - -	- - -
0.1 µL/mL	+++	++ -
Control (0 µL/mL)	+++	+++

It is probable that the much of the reduction in coagulase activity of the cells is due to a prevention of secretion of the proteins as a result of changes in the physical nature of the staphylococcal cytoplasm membrane (Shah et al., 2008).

CONCLUSIONS

The results obtained in this study show high biological potential of essential oils, particularly, the anti - *S. aureus* activity of *Origanum heracleoticum* essential oil. The essential oil studied here provided an interesting inhibitory effect of the cell viability of *S. aureus* and strongly interfered on coagulase activity. In addition, our results support the possibility of the use of *Origanum heracleoticum* derivatives as potential alternative antimicrobial compounds to be applied in food conservation, since this essential oil is considered toxicologically safe. However, further research is needed to verify its antimicrobial effectiveness in food matrices, as well as to evaluate its effectiveness to protect foods against pathogen and spoiling microorganisms throughout shelf-life.

ACKNOWLEDGEMENTS

These results are part of a research on the Project III 46012 financed by the Ministry of Education and Science, Republic of Serbia.

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NOVEL NUTRACEUTICAL MEAT PRODUCTS CONTAINING LAURICIDIN AS A DIETARRY SUPPLEMENT WITH ANTIMICROBIAL PROPERTIES

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ABSTRACT: The novel nutraceutical foods are today in the focus of the research that refers to healthy food and concerns both the food engineers and the nutricionists. Different kinds of ingredients and food supplements having certain bioregulating function, such as vitamins, antioxidants or antimicrobials, are nawadays readily incorporated in new kinds of funcional foods that are becoming more and more attractive for the consumers of healthy food. The partial glyceride monolaurin that is well known as a food additive with emulsifiing properties is also known for its antimicrobial and antiviral activity. So, it is having both nutritional and health properties that are supporting the immune system of the organism in a unique way. The monolaurin is also known as a food supplement by its commercial name Lauricidin. The aim of the study presented in this paper was to investigate the possibilities for incorporation of this food supplement in emulsified meat products and to evaluate its effect on the sensory characteristics of the products. For this purpose model meat sausages containing different commonly used additives were prepared and sensory characteristics of the different sausage formulations were compared. The antimicrobial activity of monolaurin was also preliminary investigated.
Key words: *nutraceutical meat products, monolaurin, food additives, sensory characteristics, dietary supplement, antimicrobial activity*

INTRODUCTION

The term „Nutraceuticals“ was invented by DeFelice in 1989 and it refers to food products or food supplements that are known to have certain physiological benefit or to provide protection against certain disease. Bioflavonoides derived from fruits and vegetables or meat peptides are some of these food products with known bioactivity, nutritional and functional value (Tapas et al. 2008, Mine et al. 2010). Food additives are ingredients that give the food the attribute „healthy“ if possessing some bioeregulating function in the organism (Kabara and Marshall, 2005). All ingredients used as food additives in meat products have certain functional property. Thus, the table salt and the phosphates are added to meat products in order to enhance the water binding capacity (Keeton, 2001). The curing salt is added in order to improve the colour and thus to increase the sensory characteristics of meat (Lyon and Lyon, 2001). The table and the curing salt have another very important property, they do preserve meat products and prolong their shelf life. The ascorbic and the citric acids are known as curing accelerators and have an antioxidant property that enables quick development of the specific colour and prevent the formation of unwanted nitrosamines (Toldrá and Reig, 2007). They are also known as meat tenderizers, since the acidic conditions affect the solubility of the collagen fibbers (Al-Hajo, 2009). Spices are also widely used since they enhance some sensory characteristics like the flavour and the appearance (Toldra and Reig, 2007). Besides these most common food additives, the food emulsifiers are also being widely used in the meat industry (Moonen and Bas, 2004). Monoglycerides of fatty acids are food emulsifiers approved as GRAS (Generally Recognized as Safe) food additives by the Food and Drug Administration of the USA (Hasenhuettl and Hartel, 2008).

Those biodegradable surface active agents are very popular nowadays when the sustainability is of primarily interest in all production processes (Mladenoska, 2007). Except their function as emulsifying agents, these compounds have another bioactivity that is of great importance in the nutraceutical meat products, they have an antimicrobial and an antiviral activity (Kabara and Marshall, 2005, Batovska et al., 2009). What is even more interesting, these kind of compounds are find to be usual components of some natural foods such as mother's milk or coconut milk (Liberman et al., 2006). The effect of the monoglycerides monolaurin and monocaprilin on some pathogenic microorganisms as well as on some viruses is well documented (Řihakova et al., 2002, Skrivanova et al., 2006). Those researchers suspected that the fatty acids and the partial glycerides fluidise the fats and the phospholipids in the virus's envelope and in the microbial cell membrane and this way act inhibitory to their growth. There are some attempts to incorporate those bioactive compounds in real meat products (Mbandi et al., 2004). However, the effect of the monolaurin on the sensory characteristics of the products is not extensively studied. In the present work the effect of the food supplement Lauricidin (pure monolaurin) used as an emulsifier in meat emulsified products on the sensory characteristics of the products was investigated. Several sensory characteristic such as: flavour, juiciness, tenderness, elasticity and overall acceptability were all investigated. Several most commonly used additives such as: the NaCl, the $NaNO_2$, the ascorbic acid, the citric acid and the mix of spices were all used. Their effect on the sensory characteristics of the emulsified meat products was evaluated and compared to that of Lauricidin added in two different concentrations. The antimicrobial activity of the Lauricidin used in the meat sausages and in meat hamburgers was preliminary investigated.

MATERIALS AND METHODS

Preparation of the emulsified products

Model fresh sausages were prepared according to the process flow diagram presented by Toldrá and Reig, 2007. The Lauricidin (commercial product of monolaurin purchased from Ecological formulas (Concord, California, USA)) was added to the sausages in a concentration of 0.675 and 1.36 g per 100 g sausage. The nitrite salt ($NaNO_2$) and the table salt (NaCl) were added in a concentration of 0.016 g/100 g sausage and 2 g/100 g sausage, respectively. Ascorbic acid and citric acid were added in a concentration of 0.2 g per 100 g sausage. Mixture of spices had the following composition: NaCl 1.25%, Vegeta spice mixture 3.65% and paprika 1.46%. The sausages were drained and then pasteurized at the temperature of 80 $^{\circ}$C (72 $^{\circ}$C in the center of the sausage) in duration of 30 min. A 9-point hedonic scale (9-like extremely, 1-dislike extremely) was used to evaluate sensory attributes like: flavor, juiciness, tenderness, elasticity and overall acceptability according to Lyon and Lyon, (2001). Emulsified meat hamburgers were also prepared.

Inoculation of the sausages

The pasteurized sausages and hamburgers were inoculated by 10 mL portions of seed cultures from *A. niger* MK-15 and *S. cerevisiae* TMF (belonging to the Culture Collection of the Faculty of Technology and Metallurgy, Skopje), both with concentration of approximately 10^7 cfu/mL. The inoculums were injected by a syringe. The infected sausages were kept 72 h on 28 $^{\circ}$C. Cell enumeration of the sausage samples was performed by standard plate counts and calculated as a number of colony forming units on a gram sample. 25 g of sample was diluted in 225 mL of water and homogenized. 1 mL of this solution was inoculated on a Petri dish containing 10 mL Sabouraud agar. The agar plates were incubated at 30 $^{\circ}$C during 5 days. All examined samples were diluted 10^3 times.

RESULTS AND DISCUSSION

From the results presented on the Figures 1 it can be noticed that the different additives gave different attributes to the meat emulsified products.

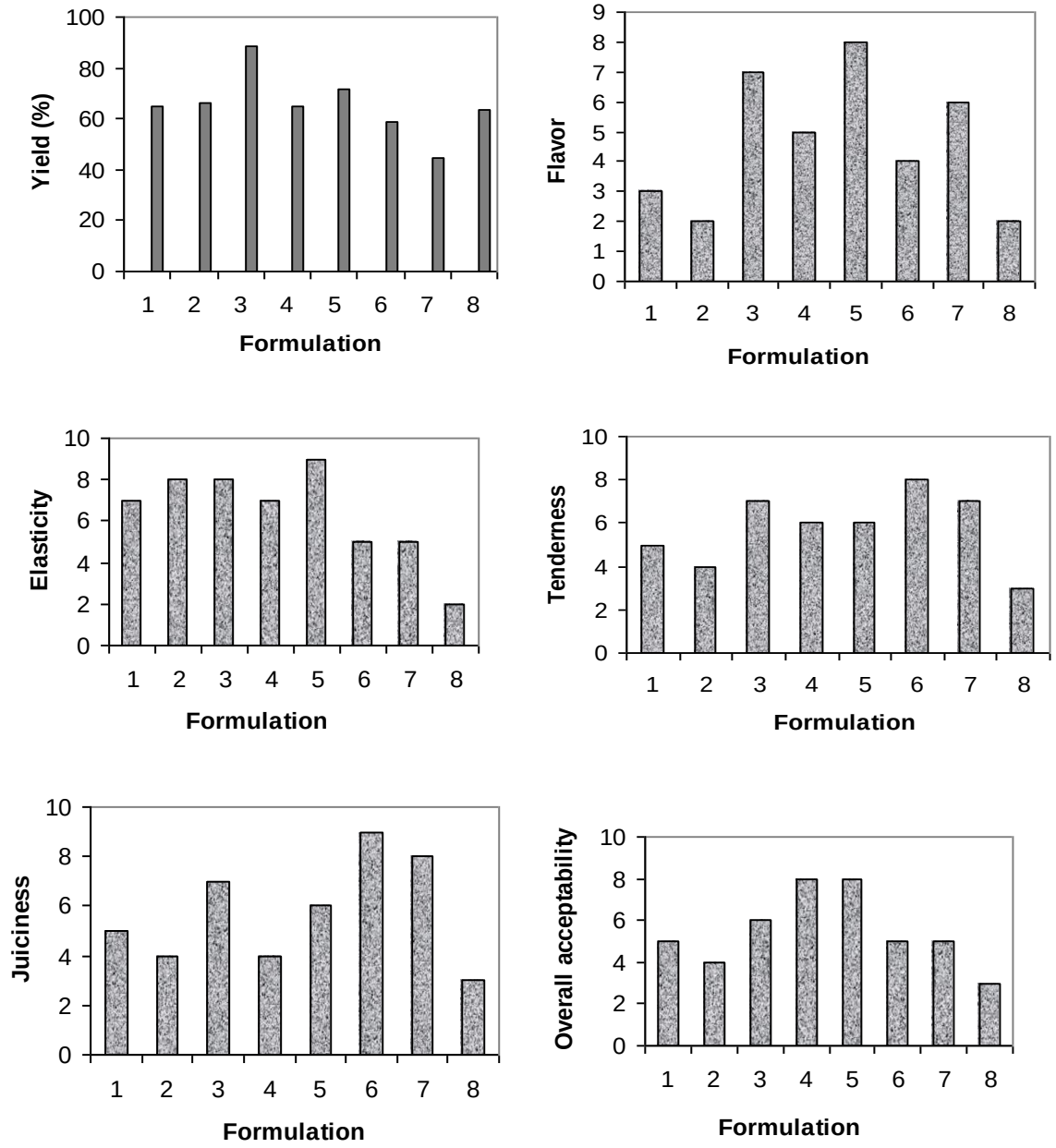

Figure 1. Sensory characteristics of the model pasteurized sausages prepared by using different kind of food additives: **1.** 0.675% monolaurin, **2.** 1. 36% monolaurin, **3.** NaCl, **4.** $NaNO_2$, **5.** mixture of spices, **6.** ascorbic acid, **7.** citric acid and **8.** additive free (the control sausage)

Thus, the mixture of spices enhanced to a great extent the scores for the product flavour and elasticity, but omitted to show high scores for the product tenderness and juiciness. The ascorbic and the citric acid, on the other hand, improved the tenderness and the juiciness of the sausages. The table salt obviously increased the water binding capacity of the meat and thereby increased the juiciness of the sausages compared to the juiciness of the additive free sausages. It was also present in a concentration of 1.25% in the spice mixture and exactly those two sausage formulations, the one with only the table salt and the one with the spice mixture, were the sausages with highest yields achieved. The negative effect of the table salt added as a sole additive was only the effect on the sausage colour (Figure 2).

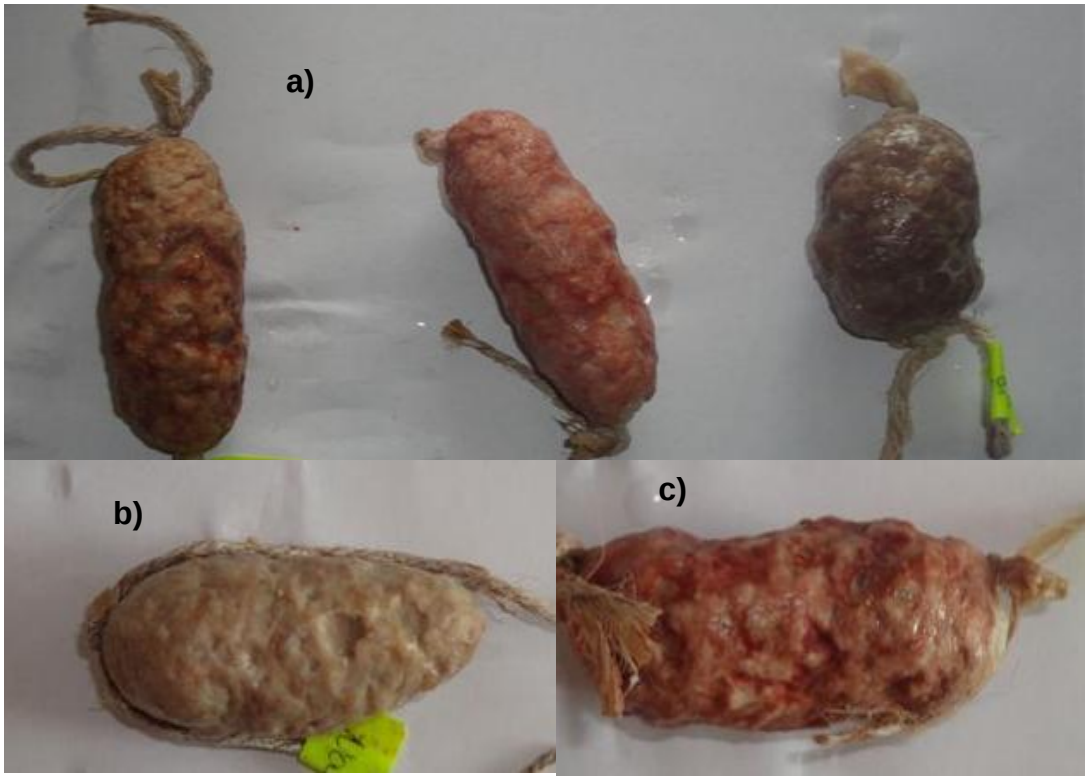

Figure 2. The appearance of the defrosted sausages after storage in a duration of 1 month: a) the additive free sausage (on the left), the sausage with $NaNO_2$ (in the middle), the sausage with ascorbic acid (on the right), b) the sausage with NaCl, c) the sausage with 0.675% monolaurin

However, the addition of the curing salt and the spice mixture, separately, had very positive effects on both the sausage colour and the flavour. Thus, the sausages that contained the curing salt and the mixture of spices yield very high scores on the sensory scale for overall acceptability.

The sausages that contained Lauricidin in a concentration of 0.675 g/100 g sausage showed good properties. They showed high scores for yield and elasticity, moderate values for tenderness and juiciness and moderate overall acceptability. However they showed very good antifungal and antiyeast activity and lowered the values for total cell count for several orders of magnitude (Figure 3).

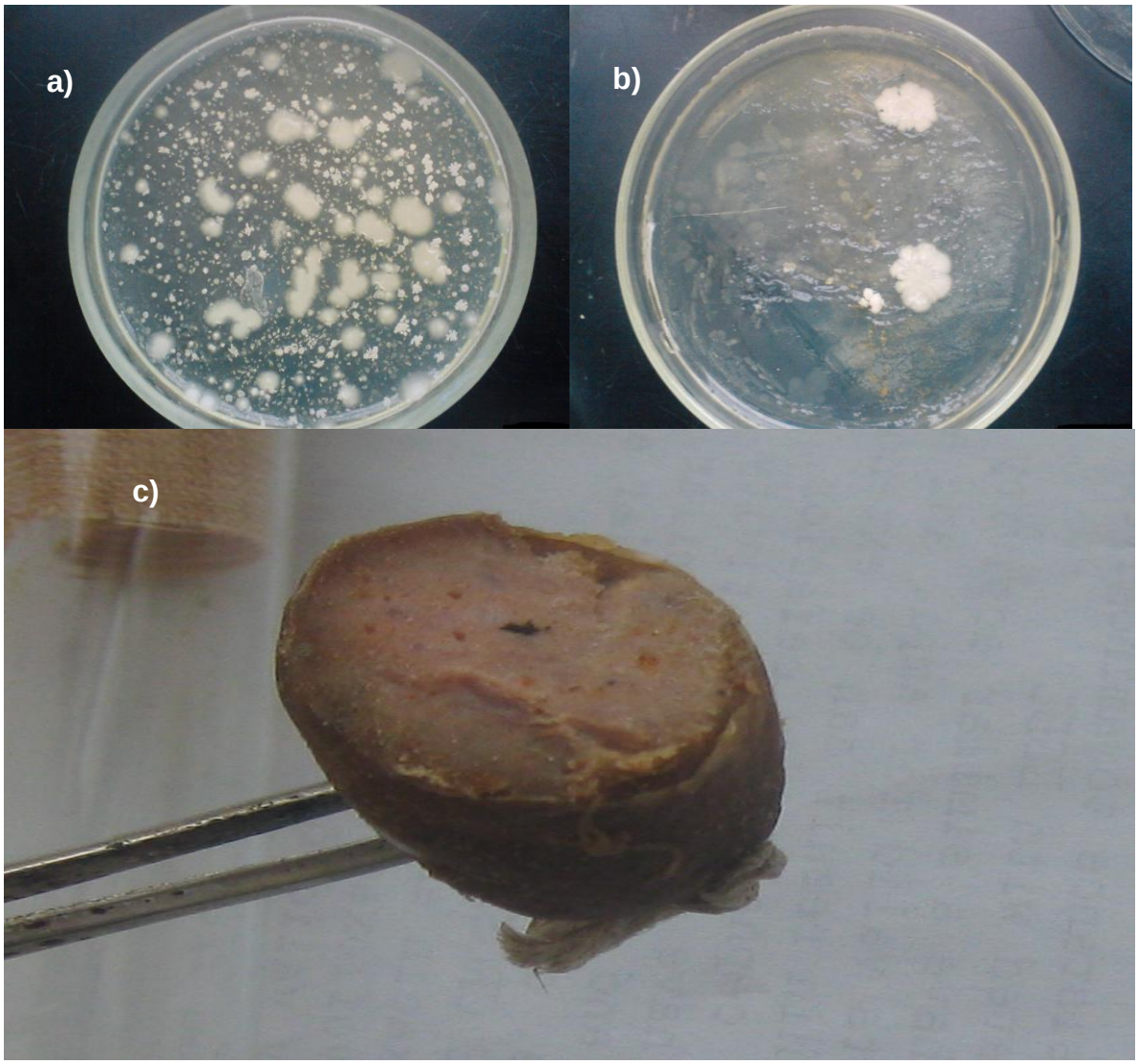

Figure 3. Growh of the *Sacharomyces cerevisiae* TMF strain isolated from the hamburgers that did not contain Lauricidin a) and form the hamburgers with Lauricidin b). c) The appearance of the cross cut of the sausage with Lauricidin infected by *Aspergillus niger* MK-15

Thus, while the total cell count of *A. niger* MK-15 calculated for the inside of the sausages that contained the monolaurin in a concentration of 0.675 g/100g sausage was only $35x10^6$ cfu/g, it's value for the inside of the sausages without monolaurin was as high as $1085 x10^6$ cfu/g. These results were in consistence with the literature data for the antimicrobial activity of the monolaurin (Řihakova et al. 2002, Kabara and Marshall, 2005). The negative effect that the monolaurin had on the product flavour (soapy taste and smell) was the only serious drawback that this additive had.

CONCLUSIONS

The food additive monolaurin could be very successfully used in meat emulsified products and give the product an attribute of healthy functional food only if it is used in low concentrations and in combination with some other food additives and spices that would mask it's soapy taste and smell.

ACKNOWLEDGEMENTS

The authors would like to thank to the Faculty of Technology and Metallurgy in Skopje, the Institute for Preventive Medicine in the General City Hospital "8th of September" in Skopje and to the Institute for Health Protection in Stip, R. Macedonia for all the help given during the research that is now a part of this work.

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OLEIC ACID CONTENT IN COW'S MILK AND VEGETABLE OILS ON SERBIAN MARKET

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ABSTRACT: Oleic acid (OA) has many beneficial effects on human health. One of the main dietary sources of OA is olive oil. Non-Mediterranean European countries, including Serbia, have low habitual olive oil consumption, but other vegetable oils, milk and diary products also contain different amounts of OA. Content of oleic and other fatty acids (FA) could markedly vary in the same foods, depending on the region. Thus the aim of this study was to determine proportion of OA in cow's milk and in eight vegetable oils available on Serbian market. FA composition of milk and vegetable oils was determined by gas chromatography. Eight vegetable oils: processed and cold pressed sunflower oil, olive oil, rapeseed oil, pumpkin seed oil, grape seed oil, linseed oil, and light sesame oil, and 5 samples of cow's milk with different fat percentages (0.5-3.5%) were analyzed. OA was found in all investigated vegetable oils in different proportions. Olive oil and rapeseed oil are main sources of oleic acid (67.0±0.4% and 64.3±0.5% of total FA, respectively), which is the least present in grape seed oil and linseed oil (18.1±0.2% and 18.7±0.1%, respectively). Pumpkin seed oil is also rich in OA (45.7±0.4%). Furthermore, significant amounts of OA were found in cow's milk (22.94-25.57% of total FA). In summary, olive oil, rapeseed oil and pumpkin seed oil are good vegetable sources of oleic acid, while cow's milk also contains considerable amounts of this FA. Regarding beneficial effects of OA, its increased consumption would lead to improvement of overall health.
Key words: *oleic acid, cow's milk, vegetable oil*

INTRODUCTION

Dietary fatty acids (FA) serve multiple functions in the body. They are constituents of cellular membranes, precursors for the biosynthesis of macromolecules, a source of energy, and are connected to both health promotion and disease pathogenesis (Bermudez et al., 2011). Therefore, the choice of dietary FA could markedly influence overall health in humans.

Emerging evidences from experimental and epidemiological studies have linked increased olive oil consumption to reduced risk of coronary heart disease, type 2 diabetes mellitus, obesity and hypertension (Kris-Etherton et al., 1999; Lopez-Miranda et al., 2010). Crucial factor in beneficial health effects of olive oil is oleic acid (OA). OA is a monounsaturated fatty acid (MUFA), 18:1n-9. Observational studies from Mediterranean cohorts using diets rich in fruits, vegetables and olive oil, have suggested that dietary OA may be protective against stroke (Samieri et al., 2011), age-related cognitive decline and Alzheimer's disease (Lopez-Miranda et al., 2010). In addition, it has been shown that insulin sensitivity was relatively impaired by diets that were low in OA, or rich in trans MUFA or saturated palmitic acid (Granados et al., 2011).

Oleic acid is found naturally in many plant sources and animal products. The main dietary source of this FA is olive oil, with the content of up to 80% of OA. It is also present in other vegetable oils, nuts, animal fats, milk and dairy products (Araujo de Vizcarrondo C, 1998; Miraliakbari & Shahidi, 2008). However, the content of OA markedly varies in the same food depending on the region.

Unlike people in Mediterranean countries who use significant amounts of olive oil in their diet, consumption of this oil is usually very low in non-Mediterranean regions, including Serbia. For this reason other dietary sources of OA are very important in these countries. The aim of

this study was to determine content of oleic acid in cow's milk and in eight vegetable oils available on Serbian market.

MATERIAL AND METHODS

Oil and milk sampling

Commercially available vegetable oils were purchased in Belgrade, Serbia, in different supermarkets and health food stores. Eight vegetable oils: processed and cold pressed sunflower oil, olive oil, rapeseed oil, pumpkin seed oil, grape seed oil, linseed oil, and light sesame oil were analyzed. Four bottles of each oil mostly from different producers were acquired for the experiment. Expiration date of all oils was at least ten months after the date of purchase. Bottles were opened prior to the experiment. Four samples of each type of oil were pooled and 10ml of the composite sample was taken for further analysis. In addition, cow's milk with 5 different fat percents (0.5%, 1.6%, 2.8%, 3.2% and 3.5%) from different producers was purchased in supermarkets in Belgrade, Serbia. Four bottles with the same percentage of fats were pooled and composite samples were analyzed.

Fatty acid determination

The total lipids from vegetable oils and milk samples were extracted according to the modified method of Folch, as we described previously (Tepsic et al., 2009). Briefly, FA were extracted from 200 µL of vegetable oil, *i.e.* from 500 µL of cow's milk using the chloroform-methanol mixture (2:1 v/v), with 50 mg% of 2,6-di-tert-butyl-4-methylphenol (BHT) added as an antioxidant. The obtained mixture was centrifuged at low speed (1000xg) to separate the two phases. The lower phase containing lipid extract was dissolved in hexane and used for further analysis. The phospholipid fraction was isolated from the extracted lipids by one-dimensional thin-layer chromatography, as we previously described (Cvetkovic, et al., 2011). Fatty acid methyl esters (FAMEs) were prepared by transmethylation with 2M NaOH in methanol (incubated at 85°C for 1h) and 1M H_2SO_4 in methanol (incubated at 85°C for 2h). Nitrogen gas was used for drying and removing solvents from the FAMEs.

FAMEs were analyzed by gas chromatography (SHIMADZU 2014, Japan,) equipped with a flame ionization detector, auto-sampler-AOC-20i and split/splitles injector. The FAMEs sample (1µL) was injected and the separation was carried out on an Optima 240 fused silica capillary column (60m x 0.25mm x 0.25µm) (MACHEREY-NAGEL GmbH& Co.KG, Germany). Helium was used as a carrier gas at pressure of 120 kPa with split ratio 1:45, and flow rate 0.32 mL/min. The oven temperature was held initially at 55ºC, increased from 210ºC at 6ºC/min and then maintained at 210ºC for 50 min. The temperature of the injection port and the detector were set at 220º and 260ºC, respectively. The fatty acids were identified by comparing their retention times with those of standards (Sigma Chemical, St Louis, MO, USA) and SUPELCO® MIX 37 component standards. All samples were determined in triplicate. The OA content was expressed as percentage of total FA.

RESULTS AND DISCUSSION

The proportion of oleic acid in total FA in vegetable oils and cow's milk are presented in Tables 1 and 2, respectively. OA was found in all investigated vegetable oils in different percentages. As expected, olive oil is the main source of OA among vegetable oils (67.0±0.4 % of total FA), but rapeseed oil had a similar content of OA as well (64.3±0.5%). Pumpkin seed oil is also rich in oleic acid (45.7±0.4%), which is also present in light sesame oil (35.1±0.1%) and sunflower oil – both processed and cold pressed (26.6±0.3 and 23.9±0.2%, respectively). The least content of OA was found in grape seed oil and linseed oil (18.1±0.2 and 18.7±0.1%, respectively). The content of the other FA of vegetable oils was also significantly different (manuscript in preparation).

With an average concentration of about 8 g/l in whole cow's milk, milk and dairy products substantially contribute to the dietary intake of oleic acid in many countries (Haug et al.,

2007). Thus we measured proportion of OA in different samples of cow's milk used in human nutrition in Serbia. As it can be seen in Table 2, we found 22.94-25.57% of OA in cow's milk, depending on the total milk fats in the samples. The proportion of OA in total FA slightly decreased with an increase of total fats in milk (from 0.5-3.5%). Regardless of small differences, OA makes around a quarter of total FA in cow's milk samples analysed in this study, suggesting that cow's milk is an important source of OA in our country. However, high consumption of milk and/or dairy products is thought to contribute to cardiovascular disease, primarily by increasing saturated fat intake, and for that reason many advisory bodies recommend avoiding high-fat dairy foods (Lichtenstein et al., 2006). Several studies have shown that the percentage of undesirable saturated FA in cow's milk can be replaced with OA by inclusion of different feed components in cow's nutrition. Komprda et al. have recently shown that feed mixture containing rapeseed, rapeseed oil and rapeseed cakes significantly decreased the content of palmitic acid and increased the content of beneficial stearic, oleic, linoleic and α-linoleic acid in milk of these cows (Komprda et al., 2005). Another study has demonstrated that supplementation of a basic diet with oilseed, linseed and sunflower seed improved the milk quality from a nutritional point of view by a large reduction in the content of saturated FA and an increase in the levels of MUFA and PUFA (Collomb et al., 2004). Thus dietary intervention in cows can contribute to healthier milk composition, particularly in terms of its fatty acid content.

Table 1. The proportion of oleic acid in total FA in vegetable oils

Vegetable oil	OA in total FA (mol%)
Olive oil	66.95±0.39
Rapeseed oil	64.35±0.49
Pumpkin seed oil	45.68±0.42
Light sesame oil	35.08±0.09
Processed sunflower oil	26.6±0.30
Cold pressed sunflower oil	23.91±0.16
Linseed oil	18.69±0.08
Grapeseed oil	18.06±0.20

Results are given as mean ± standard deviation (n = 3). OA – oleic acid, FA – fatty acid.

Table 2. The proportion of oleic acid in total FA in cow's milk samples

Cow's milk (% of fats)	OA in total FA (mol%)
0.5	25.57±0.64
1.6	25.34±0.89
2.8	23.97±0.72
3.2	23.91±1.10
3.5	22.94±0.68

Results are given as mean ± standard deviation (n = 3). OA – oleic acid, FA – fatty acid.

CONCLUSIONS

In conclusion, we have determined the proportion of oleic acid in total fatty acids in 8 vegetable oils and 5 cow's milks with different percentages of fats used in human nutrition. We have found that olive oil, rapeseed oil and pumpkin seed oil are good vegetable sources of OA. Furthermore, in non-Mediterranean countries with low habitual consumption of olive oil, cow's milk is also an important source of OA, with a content of around a quarter in total FA. Considering beneficial effects of oleic acid, its increased dietary intake would lead to improvement of overall health in Serbian population.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III41030 "Biološki mehanizmi, nutritivni unos i status polinezasićenih masnih kiselina i folata: Unapređenje ishrane u Srbiji"

(Biological mechanisms, nutritional intake and status of polyunsaturated fatty acids and folates: Improvement of nutrition in Serbia), financed by the Ministry of Science, Republic of Serbia.

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DETERMINATION OF INFLUENCE OF EXTRACTION PARAMETERS ON ANTIOXIDANT PROPERTIES OF MANDARIN PEEL EXTRACTS

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ABSTRACT: Citrus peels are a potential source of natural flavanones such as hesperidin, naringin, eriocitrin, etc. Because of their pharmacological activity and antioxidant properties, they are very interesting for pharmaceutical and food industry. In the present work, the influence of extraction parameters on the antioxidant properties of mandarin peel extracts was studied. By Taguchi experimental design four extraction parameters of conventional solvent extraction were tested: extraction temperature, extraction time, material to solvent ratio and number of stages. Antioxidant properties of mandarin extracts were determined by three different methods: radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and Antioxidant Capacity of Lipid soluble (ACL) and Water soluble compounds (ACW), which were analysed by PHOTOCHEM (Analytik Jena AG, Germany). The influence of extraction parameters on antioxidant properties was evaluated by ANOVA method. The composition and content of flavonoids in obtained extract were determined by HPLC method. Using Taguchi experimental design four extraction parameters at three levels were tested. The highest antioxidant properties were determined for mandarin extract obtained at extraction conditions: temperature 60 °C, extraction time 90 min, material to solvent ratio 1:50 g/mL and three stages of extraction. In the obtained extracts three flavanones were identified mainly hesperidin and narirutin and traces of didymin.
Key words: *conventional* solvent *extraction, mandarin peel, antioxidant properties, Taguchi experimental design*

INTRODUCTION

Citrus are an important agricultural crop of the Mediterranean area. Because citrus fruits contain several important nutrients, such as vitamin C, dietary fibre, carotenoids and flavonoids they are an important part of a healthy diet (Tripoli et al. 2007). Citrus fruits such as oranges (C. sinensis), mandarins (C. reticulata), lemons (C. lemon) and grapefruits (C. paradisi) are important for the production of fruit juices and concentrates that are mainly used in food industry for obtaining fruit drinks (Gattuso et al. 2007). There are a lot of citrus peels produced as residues, which can be a potential source of pectin and natural flavonoids (Kim et al. 2004). In citrus peels there are present two main groups of flavonoids, flavanone glycosides and polymethoxylated flavones (Tripoli et al. 2007, Ortono et al. 2006, Nogata et al. 2006). Studies have shown that citrus flavonoids play an important role in the prevention of degenerative and infectious diseases. Due to their anticarcinogenic, antiatherogenic, antimicrobial and anti-inflammatory properties flavanones and polymethoxylated flavone are very interesting for pharmaceutical and food industry (Havsteen 2002, Brunton 1999, Benavente-García et al. 1997).
Application of experimental design is very useful for estimation of the influence of different process parameters on product properties. Many different concepts are used. Genichi Taguchi developed a standard methodology where several process parameters can be tested in a defined number of experiments. By using Taguchi methodology the influence and contribution of a single parameters on the process can be studied and the optimal conditions could be determined. Therefore experimental design by Taguchi methodology is very useful

and applicable in the investigation of parameter influence on product properties (Roj 1990, Tramšek M. and Goršek A. 2007).
The aim of our investigation was to determine by Taguchi experimental design the influence of four extraction parameters (extraction temperature, extraction time, material to solvent ratio and number of stages) on antioxidant properties such as DPPH radical scavenging activity, ACL and ACW. Results were evaluated by Analysis of variance (ANOVA) method. Obtained extracts were analyzed for composition and content of flavanones by HPLC.

MATERIAL AND METHODS

Preparing of material

Mandarin peels were collected from fruits bought at the local supermarket (season 2010). Peels were dried by hot air flow (40 – 50 °C) and stored in dark and cool place. Dried peels were grounded before use and saved in dark at room temperature.

Taguchi experimental design

Taguchi experimental design was used for determining the influence of extraction parameters on antioxidant activity. Four extraction parameters, e.g. extraction temperature, extraction time, material to solvent ratio and number of stages were tested on three levels. Table 1 presents the orthogonal table of experiments L_9 prepared by Taguchi methodology (Roj 1990). For evaluation of the results Analysis of variance (ANOVA) was used. Table 2 presents the equations used for evaluation of the influence of process parameters on present antioxidant properties of obtained extracts (Roj 1990, Tramšek M. and Goršek A. 2007).
Extractions were performed at conditions presented in Table 1. The proper volume of 70 % aqueous solution of acetone was added to 5 g of mandarin peels. After the defined time of mixing by a magnetic stirrer at defined temperature the solution was separated by filtration. The extract was separated from solution by evaporation of the solvent. After drying, the extract was weighed and stored in a cool place. In the case of 2 and 3 stages extraction extract solutions were combined and evaporated to dry.

Radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH)

Antiradical activity of mandarin peel extracts was determined against stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. DPPH radical–scavenging activity of obtained extracts was determined spectrophotometically, as described by Miliauskas et al. (2004) and Majhenič et al. (2007). The radical–scavenging activity was expressed as % of inhibition of DPPH free radical.

Table 1. Taguchi experimental design for determining the influence of extraction parameters on antioxidant properties of products.

Experiment	Extraction parameter			
	Temperature (°C)	Time (min)	R(mat./sol.) (g/mL)	No. of stages (/)
1	20	120	1:20	3
2	40	90	1:20	1
3	60	90	1:50	3
4	20	90	1:30	2
5	60	120	1:30	1
6	40	60	1:30	3
7	60	60	1:20	2
8	20	60	1:50	1
9	40	120	1:50	2

Table 2: Equations used for evaluation of results by ANOVA method.

Expression	Eq.No.	Expression	Eq.No.
$S_T = \sum_{i=1}^{N} Y_i^2 - \left(\sum_{i}^{N} Y_i\right)^2 / N$	(1)	$f_e = f_T - \sum_{j=1}^{M} f_j$	(7)
$S_j = \sum_{k=1}^{L}\left(\left(\sum_{i=1}^{N_k} Y_i\right)^2 / N_k\right) - \left(\sum_{i=1}^{N} Y_i\right)^2 / N$	(2)	$f_T = M - 1$	(8)
$S_e = S_T - \sum_{j=1}^{M} S_j$	(3)	$F_j = V_j / V_e$	(9)
$V_j = S_j / f_j$	(4)	$S_j^{'} = S_j - f_j V_e$	(10)
$f_j = L - 1$	(5)	$X_j = S_j^{'} 100 / S_T$	(11)
$V_e = S_e / f_e$	(6)	$X_e = \left(S_e + \sum_{j=1}^{M} f_j V_e\right) 100 / S_T$	(12)

f_e is degree of freedom of error variance, F_j is variance ratio of process parameter j, f_j is degree of freedom of process parameter j, f_T is degree of freedom of process parameter j, L is number of levels, M is number of process parameters, N is number of experiments, N_k is number of experiments on k level, S_e is error sum of squares, S_j is sum of squares of process parameter j, $S_j^{'}$ is pure sum of squares, S_T is total sum of squares, V_e is variance of error, V_j is mean square (variance) of process parameter j, X_e is relative influence of error on optimization criteria (%), X_j is relative influence of process parameter j on optimization criteria (%) and Y_i is i value of optimization criteria, antioxidant property.

Antioxidant Capacity of Lipid soluble compounds (ACL)
Antioxidant Capacity of Lipid soluble compounds (ACL) were determined by PHOTOCHEM (Analytik Jena AG, Germany) according to the instructions of the producer. For preparation of samples (extracts) methanol was used as solvent. 10 mg of extract was dissolved in a 10 mL flask and filtrated with 45 µm Teflon filter before analysis. Extracts were measured after preparing the calibration curve of ACL standard Trolox. Results were expressed as µg of Trolox per mg of extract

Antioxidant Capacity of Water soluble compounds (ACW)
Antioxidant Capacity of Water soluble compounds (ACW) were determined by PHOTOCHEM (Analytik Jena AG, Germany) according to the instructions of the producer. For preparation of samples (extracts) MiliQ water was used as solvent. 10 mg of extract was dissolved in a 10 mL flask and filtrated with 45 µm Teflon filter before analysis. Extracts were measured after preparing the calibration curve of ACW standard Ascorbic acid. Results were expressed as µg of Ascorbic acid per mg of extract.

Flavanones composition and content in extracts
The composition and content of flavanones were determined by high performance liquid chromatography (HPLC) method. For HPLC analysis of flavonoids Agilent 1220 HPLC system with detector DAD (Agilent) and column Chomsep SS C-18 250 4.6 mm Microsorb 100 stationary phase with 5 µm particle size was used. The mobile phase consisted of two solvents: A: methanol, and B: 2 % (v/v) acetic acid in Milli-Q water. The method started with linear gradient from 35 % A to 46 % A in 30 min. The flow rate was 0.85 mL/min and detection was performed at 282 nm and 330 nm. The standard solutions were prepared by dissolving standards in methanol. Extract solutions were prepared by dissolving 10 mg of extract in 10 mL of methanol, sonicated and filtrated by 0.45 µm filter before analysis. Results were expressed as mass of single flavanone in mg per g of obtained extract.

RESULTS AND DISCUSSION

The influence of four extraction parameters (extraction temperature, extraction time, material to solvent ratio and number of stages) on DPPH radical scavenging activity, ACL and ACW was tested by Taguchi experimental design presented in Table 1. The results of antioxidant properties are presented in Table 3. All measurements were performed in triplicates. The highest activity against stable DPPH radical, 14.7 % inhibition was determined for extract obtained during extraction experiment 9: temperature 40 °C, time 120 min, material to solvent ratio 1/50 g/mL and in 2 stages of extraction. Similarly high activity against stable DPPH radical (14.4 % inhibition) was determined also for experiment 3 (60 °C, 90 min, 1/50 g/mL and 3 stages of extraction). Generally the DPPH radical scavenging of obtained extracts varied between 12.1 and 14.7 %. The highest ACL (77.26 µg of Trolox per mg of extract) was determined for extraction experiment 3, performed at 60° C, 90 min, material to solvent ratio 1/50 g/mL and 3 stage of extraction. The ACL of extracts were established between 58.78 and 77.26 µg of Trolox per mg of extract. The highest ACW of extract, 52.18 µg of ascorbic acid/mg of extract was determined for extraction experiment 9 performed at 40 °C, 120 min, material to solvent ratio 1/50 g/mL and 2 stages of extraction. The ACW values varied between 29.94 and 52.18 µg of ascorbic acid/mg of extract.
The influence of extraction parameters on presented antioxidant properties was determined by ANOVA method. High relative influence on radical scavenging activity against DPPH was determined for number of extraction stages (35.85 %) and for material to solvent ratio (34.83 %) while time and temperature of extraction were less influencing parameters (14.71 and 14.61 %). For ACL of extract all tested parameters influence at a similar extent; temperature of extraction – 24.99 %, time of extraction – 24.17 %, material to solvent ratio – 23.46 % and number of extraction stages – 27.38 %. The main influence on ACW belonged to the time of extraction (83.76 %) while other parameters (number of extraction stages, material to solvent ratio and temperature) had small influence (7.69, 6.07 and 2.48 %).

Table.3 Antioxidant properties of extracts of mandarin peel.

	DPPH	ACL	ACW
Experiment	% of inhibition	µg Trolox / mg of extract	µg Asc. acid / mg of extract
1	13.4	58.78	44.10
2	12.1	62.25	24.55
3	14.4	77.26	30.12
4	12.5	74.76	29.94
5	13.1	67.81	40.32
6	13.8	68.42	44.16
7	13.3	68.95	40.93
8	12.9	58.97	41.90
9	14.7	68.02	52.18

In a further investigation the composition and content of flavonoids were determined (Figure 1). The two main flavanones hesperidin and narirutin were determined by HPLC method. Didymin was present in traces. The content of hesperidin in extract varied between 71.2 and 110.7 mg of hesperidin per g of extract, while the content of narirutin was much lower, between 16.8 – 20.8 mg of narirutin per g of extract. The highest content of hesperidin, 110.8 mg/ g of extract, was obtained by extraction at 40 °C, 120 min, 1/50 g/mL and in 2 stages of extraction (experiment 9). Similarly high content of hesperidin (104.4 and 103.8 mg per g of extract) was determined also for extract of experiment 3 and experiment 7. The highest content of narirutin, 20.8 mg/ g of extract, was extracted at conditions: 60 °C, 60 min, 1/20 g/mL and 2 stages (experiment 7).

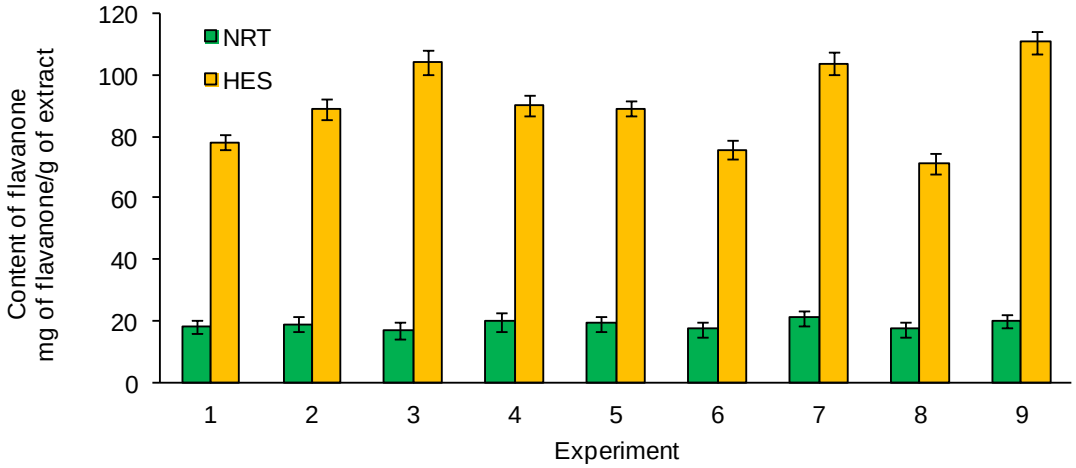

Figure 1. The content of the main flavanones hesperidin (HES) and narirutin (NRT) in extracts.

CONCLUSIONS

In the present work Taguchi experimental design was applied for the first time for evaluation the influence of different extraction parameter on antioxidant properties of mandarin peel extracts. The highest influences on the radical scavenging against DPPH have the number of extraction stages and material to solvent ratio. All studied parameters have similar influence on ACL property. The material to solvent ratio has predominating influence on ACW property of extracts.

The presented methodology can be applied also for the determination of the optimal extraction conditions to obtain extracts with the best antioxidant properties. Two very good combinations of tested antioxidant properties were determined for extracts obtained at extraction experiment 3 (60 °C, 90 min, 1:50 g/mL and 3 stages of extraction) and experiment 9 (40 °C, 120 min, 1/50 g/mL and 2 stages of extraction). In the obtained extracts three flavanones were identified: mainly hesperidin and narirutin and traces of didymin.

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OLIGOSACCHARIDES IN LEGUME GRAINS

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ABSTRACT: Oligosaccharides from legume species produced in few regions of Croatia were investigated. Eight traditionally cultivated legumes were analyzed: common bean *(Phaseolus vulgaris* L.*),* faba bean *(Vicia faba* L.*),* lentil *(Lens culinaris* Medic.*),* white lupin *(Lupinus albus),* chickpeas *(Cicer arietinum* L.*),* cowpea *(Vigna unguiculata* L.*),* soybean *(Glycine max* (L.) Merr.*)* and grass pea *(Lathyrus sativus).*The soluble saccharides (monosaccharides, sucrose, raffinose and stachyose) were extracted with water and separated by high performance liquid chromatography. Saccharides were identified by their retention time and quantified by peak area using external standard procedure.The results showed that the total water soluble saccharides content ranged from 3.71% to 7.5%, and oligosaccharides represented 36.38% to 69.29% of the total saccharides in investigated dry legume grains. Stachyose was the main oligosaccharide in all grains, except for chickpeas, in which the main oligosaccharide was raffinose. Different amounts of sucrose and small amount of glucose, galactose and fructose were present in all legume grains. Significant difference in total saccharides, raffinose and stachyose content was found between investigated legume species. The discriminant analysis showed that legume species can clearly classify according to content of water soluble saccharides. According to obtained results, the investigated legumes can be considered a good source of oligosaccharides.
Key words: *legume species, oligosaccharides, HPLC separation*

INTRODUCTION

Oligosaccharides are functional food ingredients that have great potential to improve the quality of food. Oligosaccharides have been associated with many health-promoting functions, which had been identified in many clinical studies, such as promoting the growth of *Bifidobacterium* in human intestine and balance of intestinal bacteria, modulation the immune response, inhibition of cancer and tumour and stimulation of mineral absorption (Mesina, 1999).

Legume grains are a rich source of galactooligosaccharides, namely raffinose and stachyose: raffinose is a trisaccharide containing a galactose linked -(1-6) to the glucose unit of sucrose; stachyose is a tetrasaccharide containing a galactose linked -(1-6) to the terminal galactose unit to raffinose. Humans cannot digest these oligosaccharides because they do not possess the enzyme α-galactosidase necessary for hydrolysing the linkage present in oligosaccharides in consumed food. Intact oligosaccharides reach the colon, where they are preferentially fermented by beneficial bifidogenic microorganisms that contain the enzyme. Fermentation of nondigestible oligosaccharides results in production of gases and short chain fatty acids, which are interesting because of their prebiotic activity associated with health benefits (Roberfroid and Slavin, 2000). Oligosaccharides potential as an ingredient of functional food makes the search for new sources interesting, as well as the development of methods that allow its isolation and purification in a simple and effective way (Guillon and Champ, 2002).

The objective of this study was to investigate the composition of oligosaccharides in legume species produced in few regions of Croatia.

MATERIAL AND METHODS

Eight traditionally cultivated legumes: common bean (*Phaseolus vulgaris* L.), faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medic.), white lupin (*Lupinus albus*), chickpeas (*Cicer arietinum* L.), cowpea (*Vigna unguiculata* L.), soybean (*Glycine max* (L.) Merr.) and grass pea (*Lathyrus sativus*) were used for analysis.
To prepare a sample for analysis, one gram of ground legume was weighed into a test tube and mixed with water. The tube was placed horizontally on a shaker for 20 min at room temperature. After cooling, sample was centrifuged and 5 mL of the clear supernatant was transferred into a new test tube. A total of 7 mL of acetonitrile was added to precipitate soluble proteins and incubated at room temperature for two hours. The mixture was then centrifuged and the supernatant was collected and evaporated to dryness in a rotatory evaporator. The dried extract was disolved in water and passed through 0.45 μm syringe filter, just before high performance liquid chromatography (HPLC) analyses. The separation was carried out by Perkin-Elmer High-Performance Liquid Chromatography system series 200 equipped with degasser, isocratic pump, oven, refractive index detector and TotalChrom Navigator (HPLC software). The separation was performed on MetaCharb Ca Plus column (300 x 7.8), thermostated at 90^0C. 20 μL aliquot was injected onto the column and eluted with deionized water of flow rate of 0.5 mL/min. A standard solution composed of stachyose, raffinose, sucrose, glucose, galactose and fructose at concentration of 1; 2; 2.5; and 3 mg/mL respectively. Saccharides from aqueous sample extract were identified by their retention time and quantified by peak area using external standard procedure. Oligosaccharides were representeed as the sum of raffinose and stachyose.
One-way analysis of variance (ANOVA) and multiple comparisons (Duncan's post hoc test) were used to evaluate the significant difference of the data at $P < 0.05$. Comparative analyses of legume were performed using linear discriminant analysis. All statistical analyses were performed using statistical-graphic system "Statistica" version 7.0 (Stat Soft software Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

The composition of water soluble saccharides in legume grains, determined by HPLC, is shown in Fig.1 and 2. The results indicate that the legume grains contain oligosaccharides, disaccharide and monosaccharides.

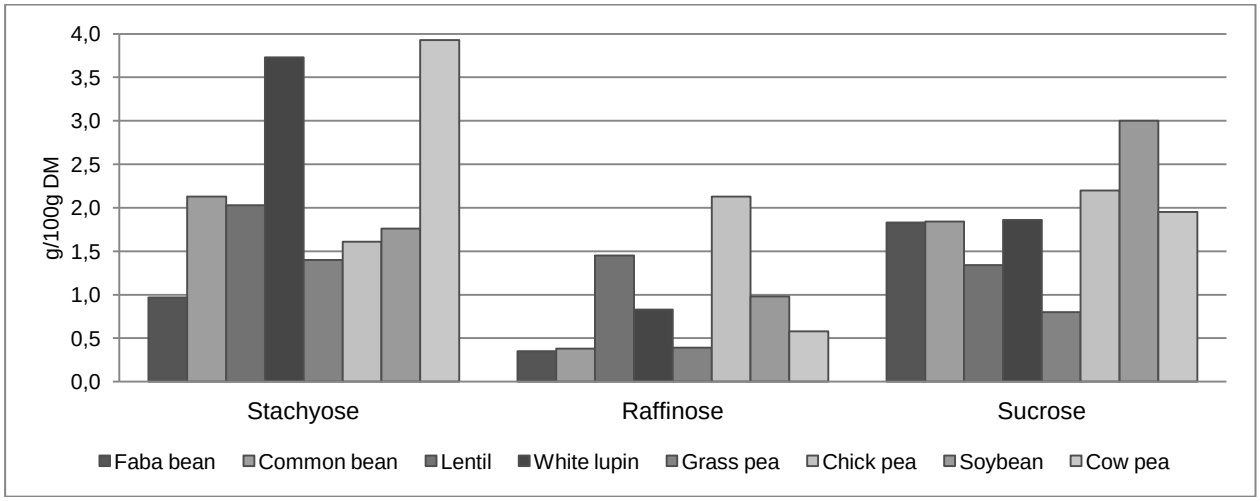

Figure 1. Concentrations (%, dm) of stachyose, raffinose and sucrose in legume grain

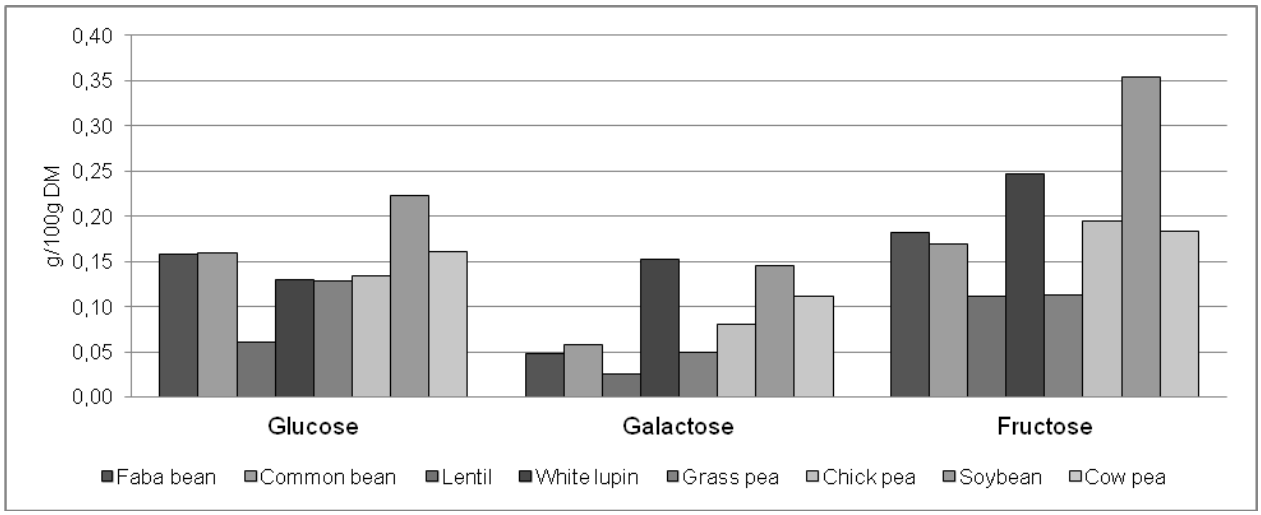

Figure 2. Concentrations (%, dm) of glucose, galactose and fructose in legume grain

Most dominant oligosaccharide was stachyose. Among legume species, high level of this oligosaccharide was found in cow pea (3.93%) and white lupin (3.73%), and the least in faba bean (0.93%). Stachyose has been reported as main oligosaccharide in most legumes, i.e. lentils, chickpeas, common bean and white lupin (Kueo et al., 1988; Guillon and Champ, 2002; Berrios et al., 2010; Hou et al., 2006). The content of stachyose detected in this legume was within the range 2.21-3.23 % reported by Pilar (1998). Wide range of stachyose was found in soybean (Wilcox and Shibles, 2001; Kim et al., 2003; Espinosa-Martos and Ruperez, 2006; Giannoccaro et al., 2008). Hou et al. (2010) analyzed worldwide soybean germplasm and found stachyose content ranged from 0.2 to 69.6 mg/g and several samples contained less than 10 mg/g. Raffinose amount was highest in chick pea (2.13%) and lowest in faba bean (0.35%), common bean (0.38%) and chick pea (0.39%). Sucrose, as only disaccharide found, was the second most abundant water soluble saccharide, with amounts ranging from 0.80% (grass pea) to 3.00% (soybean). In the soybean, sucrose is dominant sugar ranging from 3% to 10% and it is responsible for the sweet taste of soy food (Giannoccaro et al., 2008). Small amounts of monosaccharides: glucose, galactose and fructose (<1%) have also been found (Fig. 2). Besides small but different amounts of glucose and fructose, it is interesting to note that galactose, not previously detected in legume, was found in all species.

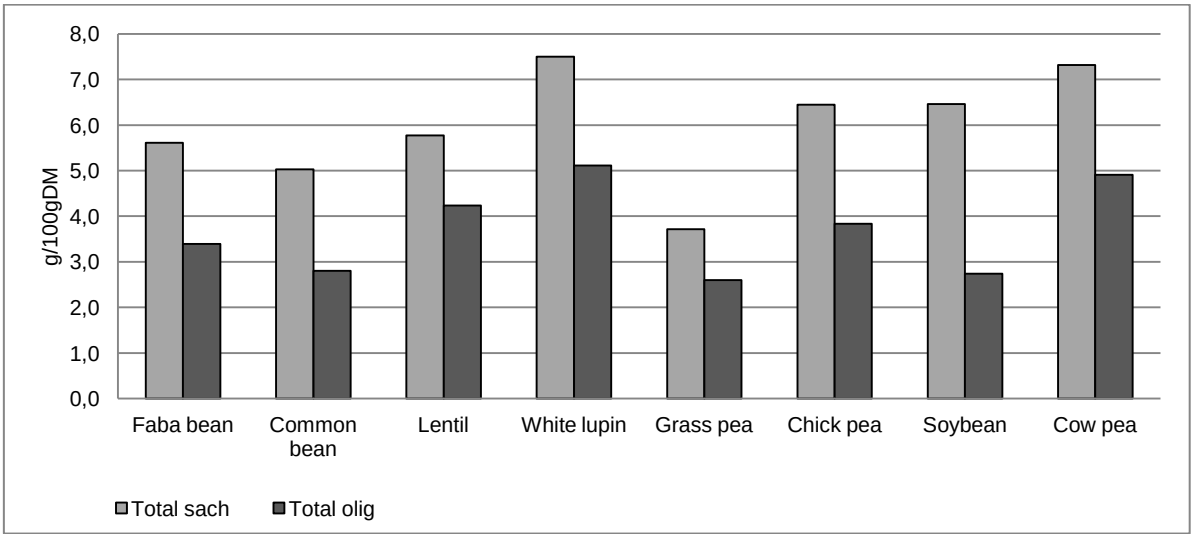

Figure 3. Concentration (%, dm) of total saccharides and oligosaccharides in legume grain

The content of total water soluble saccharides ranged from 3.71% (gras pea) to 7.50% (white lupin), and oligosaccharides represented 36.38% to 69.29% of the total saccharides in investigated dry legume grains (Fig. 3). In this study, the results of total water soluble saccharides and oligosaccharires in legume species were in agreement with some published data (Ruperez, 1998; Han and Baik, 2006). The analyses of the saccharides profiles indicated that high total saccharides content is attributed to high content of sucrose and oligosaccharides.

Table 1. Results of ANOVA for total water soluble saccharides in legume species

EFFECT	DF	Stach	Raf	Suc	Glc	Gal	Fru	Total sacch	Total oligo
Total	75	0.84	0.33	0.52	0.00	0.00	0.01	2.09	0.87
Blocks	3	0.00	0.00	0.01	0.00	0.00	0.00	0.04	0.01
Species	7	8.38**	3.43**	4.63**	0.01**	0.02**	0.05**	17.79**	7.99**
Residual	65	0.07	0.01	0.10	0.00	0.00	0.00	0.50	0.15
C.V.		13.10	16.30	18.10	29.90	28.01	16.06	12.90	11.41

Analysis of variance showed significant difference ($p<0.05$) between investigated legume species for: stachyose, raffinose, sucrose, monosaccharides, total saccharides and oligosaccharides content (Tab. 1).
Individual saccharides content as the variables in discriminant analysis were used for classification and differentiation of legume species.

Table 3. Results of cannonical discrimant analysis on legume species

Variable	Pooled with cannonical structure		
	Can1	Can2	Can3
Stahiose	-0,17	0,48	-0,47
Raffinose	0,61	0,31	-0,19
Sucrose	0,12	-0,06	-0,45
Glucose	-0,01	-0,08	-0,14
Galactose	0,01	0,02	-0,43
Fructose	0,09	-0,57	-0,57
Variation %	54,65	26,01	16,32
Total variation %	54,65	81,66	97,98
Discriminate r>±0,40	Raffinose	Stahyose Fructose	Sucrose Galactose

All multivariate difference tests between legume groups were significant on the 0.001 level and the credibility test indicated significance of the first three out of six newly created canonical variables. The first three variables explained 97.98 % of the total variance (Table 3.) with the square canonical correlation percentage of R^2= 80.51%. The first canonical variable (54.66%) had the strongest legume group discrimination through raffinose influence. The raffinose content (0.61) proved to be a trait that discriminates population the most and clearly differentiates legume species. In the second canonical variable stachyose and fructose dominated. These are variables which are similar in influence but of different directions.

CONCLUSION

In investigated legume grain wide variation were found among individual and total water soluble saccharides. Stachyose was the main oligosaccharide in all grains, except for chickpeas, in which the main oligosaccharide was raffinose. Sucrose was the second most abundant water soluble saccharide, while glucose, galactose and fructose were presented in small quantities. According to obtained results, the investigated legumes can be considered a good source of oligosaccharides for functional food. The discriminant analysis showed that

the legume species can be clearly classified according to content of water soluble saccharides.

ACKNOWLEDGMENT

This research was supported by SEERANET 168 (Sustainable preservation of indigenous South East European legumes and their traditional food and feed products) project. We wish to thank the project Continued genetic improvement of soybean by modern breeding strategies (073-0730489-0344) for the financial support.

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PGSS™ FORMULATION AND CHARACTERIZATION OF YELLOW PIGMENT EXTRACT FROM CURCUMA

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ABSTRACT: In the present work the curcumin extract was formulated using supercritical fluid technology, namely Particles from Gas Saturated Solution technique (PGSS™). The PGSS™ formulation was carried out using supercritical carbon dioxide, which results in an organic solvent-free powderous product. The investigated carrier materials were fat and different natural polymers.
Powderous products obtained after the PGSS™ micronization showed good colour stability, which gives good bases for potential applications in the future. The products are homogenously coloured fine powders with colours from light yellow to orange; what depends on contents of curcuminoids.
The radical-scavenging activities of powderous products was evaluated using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). All powderous products displayed radical-scavenging properties.
Key words: *curcumin, PGSS™, DPPH*

INTRODUCTION

One of the newest trends in food science and technology is functional food. Curcumin, a natural yellow-orange polyphenol found in the plant *Curcuma longa* (turmeric), is widely used as a food colourant and as a potential protective agent against several chronic diseases, including cancer, HIV-infection, neurological, cardio-vascular and skin diseases (Paramera et al., 2011). It has a wide range of pharmacological activities including anti-inflammatory, antioxidant, antiproliferative and antiangiogenic properties. Curcumin is unstable at neutral and basic pH values. It would be stable in the stomach and small intestine since degradation of curcumin is extremely slow at pH between 1 and 6 (Setthacheewakul et al., 2010).
The aim of this study was to formulate powdereous products from curcuma and test their antioxidant activity.

MATERIAL AND METHODS

Curcumin liquid extract (10 wt. %) was purchased from Etol (Celje, Slovenia). CO_2 was provided by Messer (Ruše, Slovenia).

Formulation with PGSS™

Curcumin extract was first formulated with various carriers using supercritical fluid technology, namely Particles from Gas Saturated Solution technique (PGSS™). The melted fat (glycerol tristearate) was mixed with the emulsifier and the curcumin extract in different concentrations using a homogenizer. Then pure cellulose or pure starch was added to the mixture to make better texture of the products. The autoclave was filled with the substance and CO_2 was introduced by a high pressure pump to the desired pressure, depending on prexpansion parameters (160 bar). The autoclave was then heated up to the operating temperature which was slightly higher than the melting point of the fat (~60 °C). Simultaneously the pressure reached the operating value. The autoclave with its content was shaken constantly until reaching the equilibrium (approximately 2 h). The solution saturated with gas was then expanded through the nozzle and the compressible gas evaporated

instantly in the expanding chamber causing the micronization of the particles (Mandžuka et al., 2010).

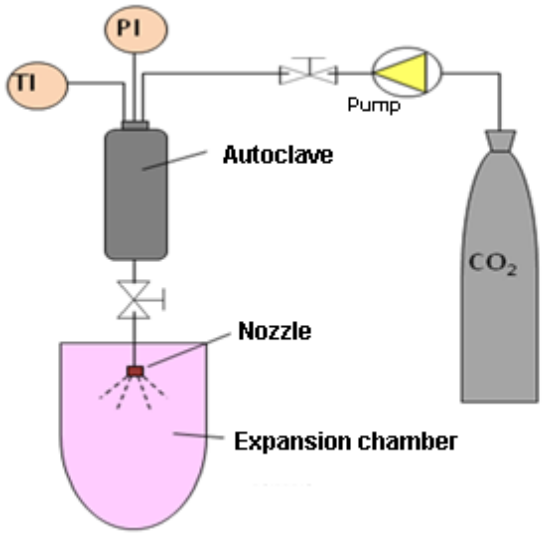

Figure 1. Scheme of PGSS^{TM} equipment

DPPH radical scavenging activity

DPPH radical-scavenging activity of the curcuma extract was measured with the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich, Slovenia) free radical. The reduction capability (on the DPPH radical) is determined by the decrease in its absorbance at its absorption maximum at 515 nm that is induced by antioxidant. This is visualized as a change in colour from violet to yellow. Extract solutions were prepared by dissolving 0.01 g of micronized powder in 10 ml of methanol. The solution of DPPH in methanol was prepared daily, before UV measurements. 3 mL of this solution were mixed with 77 µl extract solution in the flask. The samples were kept in the dark for 15 min at room temperature and then absorbance was measured at 515 nm. Radical-scavenging activity was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{\quad}{\quad}$$

where *Ab*, absorption of blank sample (*t=0* min); *Aa*, absorption of tested extract solution (*t=15* min) (Al-Reza et al., 2010).

RESULTS AND DISCUSSION

Results of micronizations

Table 1 shows the composition of samples and the operating parameters applied for the formulations. The obtained curcuma products were homogenously coloured fine free-flowing powders with colours from light yellow to orange.

The used operating parameters were about 170 bar and 70 °C in all experiments. By using cellulose or starch the texture of obtained powder was better and drier in all cases.

Results of antioxidant activity

The results of DPPH radical-scavenging activities of the micronized powders are represented in Figure 1. The micronized powders showed potent DPPH radical - scavenging activities in range between 6.98 to 14.46 % and are applicable as colorants in food or pharmaceutical industry. The highest radical-scavenging activity of 14.46 % was observed for sample 3 (50 % curcumin extract, 45 % glycerol tristearate and 5 % pure cellulose). The lowest radical - scavenging activities were observed for sample 1 and 2 (20 % curcumin extract, 65 % glycerol tristearate and 5 % pure cellulose or starch).

Table 1. Results of formulation of curcumin extract

Samples	Curcumin extract (%)	Emulsifier (%)	Glyc. trist. (%)	Cellulose (%)	Starch (%)	p (bar)	T (°C)	Yield (%)	Product
1	20	1	65	15	-	160	64	77.7	
2	20	1	65	-	15	160	63	92.4	
3	50	1	45	5	-	160	70	90.6	
4	50	1	45	-	5	160	70	75.1	
5	50	1	30	20	-	160	60	80.2	
6	50	1	30	-	20	160	59	70.6	

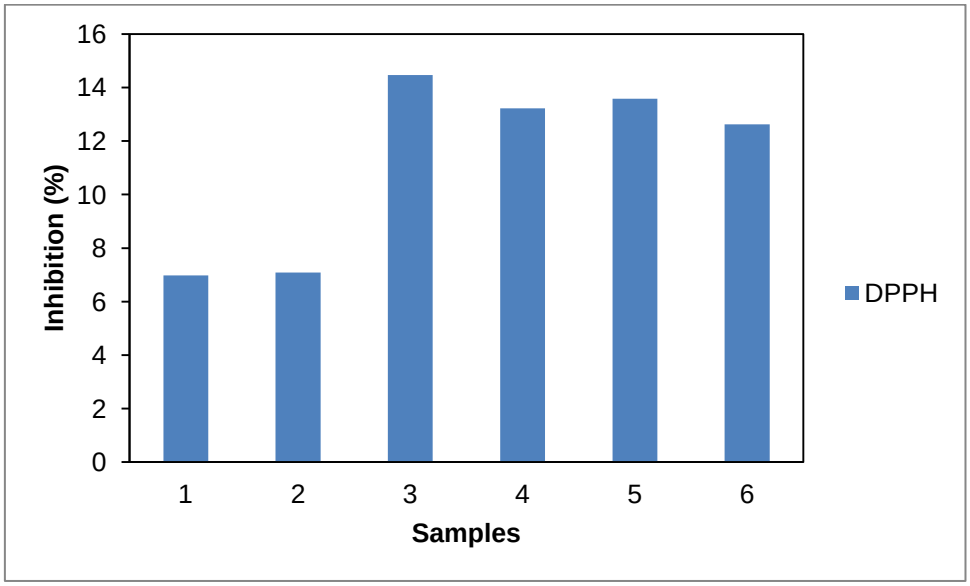

Figure 1. Antioxidant activities of the micronized powders

CONCLUSIONS

In this work application of environmentally friendly technology was applied for the formulation of curcuminoids from curcumin extract in the powderous form. The obtained dense curcumin extract was formulated in high pressure process (PGSS^{TM}) by using CO_2 as processing media and glycerol tristearate fat mixed with cellulose or starch as carriers.
The obtained products were homogenous fine powders with colours from light yellow to orange with high radical-scavenging properties. The obtained products are interesting for the application in the food industry.

ACKNOWLEDGEMENTS

Authors are grateful to the Slovenian Ministry of High Education, Science and Technology for the financial support of this work.

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PHENOLIC PROFILE, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF OREGANO (*Origanum vulgare* L., LAMIACEAE) POSTDISTILLATION WASTE EXTRACTS

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ABSTRACT: Aromatic herbs have long tradition of use in medicine, nutrition and cosmetics. Among them, oregano (*Origanum vulgare* L., Lamiaceae) has been appreciated as a spice and food flavouring agent owing to the presence of essential oil. However, postdistillation waste, remaining after the isolation of essential oil, is still unused. Thus, in this study antioxidant and antimicrobial activity of oregano herb postdisillation waste material was investigated. After the hydrodistillation of essential oil of oregano herb, postdistillation waste material extracts were made from decoction and used herb (with 45% v/v and 75% v/v ethanol). Chemical composition was determined by means of high performance liquid chromatography. *In vitro* scavenging activity was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazile) ($DPPH^{\cdot}$) and hydroxyl ($OH^{\cdot}$) radical. The effect on the peroxidation of membrane lipids was assayed by thiobarbituric acid (TBA) test, following the effect of investigated extracts on Fe^{2+}/H_2O_2 induced lipid peroxidation (LP) in corn oil as a model system. Minimal inhibitory concentration (MIC) was determined with broth microdilution assay against selected gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) and gram-negative bacteria (*Salmonella* Infantis, *Escherichia coli*). All investigated extracts were rich in phenolcarboxylic acids (rosmarinic, protocatechuic, gallic and chlorogenic acid) and flavonoids (rutin and apigenin). They exhibited strong antioxidant and antibacterial activity (especially extract obtained with 75% ethanol from used herb) when compared to standardised oregano herb extract. This study confirms postdistillation plant waste material for possible use in pharmaceutical and food industry and gives very informative insight into antioxidant and antibacterial profile of investigated oregano extracts.
Key words: *Oregano, postdistillation plant waste material, phenolics, antioxidant, antibacterial*

INTRODUCTION

Many species of *Oregano* genus, native to central and southern Europe, the Balkans and Asia are widely used as culinary and medicinal herbs (Bisset and Wichtl, 2001). *Origanum vulgare* L., (Lamiaceae) is the most frequently used and cultivated worldwide. Numerous studies confirmed antioxidant, antibacterial, spasmolityc and carminative effect for the essential oil (Dorman and Deans, 2000; Burt, 2004). Oregano is widely used as spice in food processing and in many pharmacological preparations as herbal remedies (Tepe et al., 2004). Besides essential oil, various oregano extracts (mainly ethanol) have been studied for their antioxidant and antimicrobial activity in various commercial or food models (Chun et al., 2005; Hernandez-Hernandez et al., 2009; Camo et al., 2011). It is considered that high content of phenolic compounds in oregano, especially caffeic acid oligomers (e.g. rosmarinic acid) and flavonoid glycosides contributed the most to the radical scavenging activity of the herb and investigated extracts (Gomez-Estaca et al., 2009). The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play an important role in neutralizing free radicals, chelating transitional metals and quenching singlet and triplet oxygen, by delocalization or decomposing peroxides.

Moreover, phenolic compounds from plant extracts have been used in different food matrices to improve stability of food lipids. Oregano herb contains minimum 2.5% of essential oil and it is mainly used for its hydrodistillation (Ph. Eur. VI, 2007). As a result, majority of plant material remains unused.
Considering these facts, the aim of the present study was to evaluate antioxidant and antimicrobial potential of chemically well characterized oregano herb postdistillation waste material.

MATERIAL AND METHODS

Plant material

Oregano herb (*Origanum vulgare* L., Lamiaceae) was obtained from the Institute for Studies on Medicinal Plants „Dr Josif Pancic" in Belgrade in 2011. Voucher specimen of purchased plant material (oregano no. Ov-16/10) was confirmed and deposited at the Herbarium of the Laboratory of Pharmacognosy, Department of Pharmacy, Faculty of Medicine, University of Novi Sad (Tutin et al., 2001).

Extraction procedure

Oregano herb was macerated with 45% ethanol (EtOH) for 24 h at room temperature (1:10 w/v, 10 g dried leaves) to prepare standard extract 1 (E1). Essential oil was isolated by hydrodistillation, and decoction remained after this procedure was used to prepare extract 2 (E2). Deodorized extracts were obtained after distillated plant material was dried and re-extracted with 45% (E3) and 75% (E4) ethanol using cold maceration process. All extracts were filtered and evaporated to dryness under vacuum. For the evaluation of total phenolic and flavonoid content and antioxidant activity, residues were immediately dissolved in water to make 10% (w/v) stock solutions. For HPLC analysis residues were dissolved in methanol:1% formic acid mixture (50:50 w/w) to make 10% (w/v) stock solutions. For antibacterial testing residues were reconstituated in solvents used for extract preparation (water, 45% or 75% EtOH).

Chemical composition

Determination of total phenolic content: The amount of total phenolic compounds in extracts was determined spectrophotometrically with Folin-Ciocalteu (FC) reagent (Lakic et al., 2010). The concentration of total phenolic compounds was expressed in mg of gallic acid equivalents (GAE) per g of dry extract (d.e.) using a standard curve of gallic acid (concentration range 0.08-0.24 mg/mL). All measurements were done in triplicate.

Determination of total flavonoid content: Total flavonoid content in the extracts was determined spectrophotometrically, using a method based on the formation of a flavonoid-aluminium complex with an absorptivity maximum at 430 nm (Jia et al., 1999). Flavonoid content was expressed in mg of quercetin equivalents (QE) per g of dry extract using a standard curve of quercetin (concentration range 10-100 µg/mL). All measurements were replicated three times**.**

HPLC analysis: HPLC analysis was performed using a liquid chromatograph (Agilent 1200 series), equipped with diode array detector (DAD) and Eclipse XDB-C18, 1.8 µm, 4.6×50 mm column, at a flow-rate of 1 mL/min. Solvent gradient was performed by varying the proportion of solvent A (methanol) to solvent B (1% formic acid in water (v/v)) (Misan et al., 2011). The total running time and post-running time were 45 and 10 min, respectively. The column temperature was 30 ºC. The injected volume of samples and standards was 5 µL and it was done automatically using autosampler. The spectra were acquired in the range 210–400 nm and chromatograms plotted at 280, 330 and 350 nm with a bandwidth of 4 nm, and with reference wavelength/bandwidth of 500/100 nm.

Antioxidant activity

Free radical scavenging capacity (RSC) of the extracts was evaluated by measuring scavenging activity on DPPH and OH radicals and combined with determination of influence on lipid peroxidation for the assessment of antioxidant activity. All measurements were done in triplicate. Percent of RSC or inhibition of lipid peroxidation process was calculated by the following equation:

$$RSC/I\ (\%) = 100\% \times (A_{control} - A_{sample})/A_{control}$$

From the obtained RSC/I (%) values the IC_{50} value, which represents the concentration of an extract that caused 50% neutralization or inhibition, was determined by regression analysis.

DPPH assay: DPPH assay was performed as described previously (Bozin et al., 2008). The disappearance of DPPH radical (DPPH$^{\bullet}$) was detected spectrophotometrically at 515 nm.

To standardize DPPH results, the antioxidant activity index (AAI) was calculated as follows:

$$AAI = \text{DPPH concentration in reaction mixture } (\mu g/mL)/IC_{50}\ (\mu g/mL)$$

Samples were classified as showing poor antioxidant activity AAI<0.5; moderate 0.5<AAI<1; strong 1<AAI<2 and very strong AAI>2 (Sherer and Godoy, 2009).

Neutralisation of OH radical (OH$^{\bullet}$): The scavenging capacity of oregano extracts for OH$^{\bullet}$ was evaluated by measuring the degradation of 2-deoxy-D-ribose with OH$^{\bullet}$ generated in the Fenton reaction (Bozin et al., 2006).

Inhibition of lipid peroxidation: The extent of LP was determined by measuring the colour of the adduct produced in reaction of TBA with malondyaldehide (MDA), as the final oxidation product in the peroxidation of lipids (Milic-Torres et al., 2011). After cooling and centrifugation at 3500r/min for 20 min, the content of the MDA was determined by measuring the absorbance of the adduct at 532 nm (test tubes were kept at 0 °C during measurement).

Antibacterial testing

Bacterial strains and growth conditions: Four bacterial strains, namely *Bacillus cereus* WSBC 10530 (clinical isolate), *Staphylococcus aureus* ATCC 25923 (clinical isolate), *Salmonella* Infantis ŽMJ 106 (poultry meat isolate) and *Escherichia coli* O157:H7 ŽM 370 (clinical isolate) were used for antibacterial testing. The cultivation/assay medium for used bacterial strains was Müeller Hinton Broth or Agar (MHB, MHA, Oxoid, Hampshire, UK). For antimicrobial testing, bacterial cultures were prepared by picking colony from 24-h-old MHA plates and they were suspended in an appropriate medium (MHB) (5 mL). Cultures were grown aerobically for 24 h and continuously shaken at 100 rpm at 37 °C. For antibacterial activity assays 1 mL of each culture was diluted with MHB medium to 10^6-10^7 CFU/mL.

Broth microdilution method: For the broth microdilution test 50 µL of each bacterial suspension in suitable growth medium was added to the wells of a sterile 96-well microplate already containing 50 µL of two-fold serially diluted investigated extracts. The final volume in each well was 100 µL. Control wells were prepared with culture medium, bacterial suspension only, extracts only and ethanol in amounts corresponding to the highest quantity present. After the incubation period of 24 h, minimal inhibitory concentration (MIC) values were determined as the lowest concentration where no viability was observed on the basis of metabolic activity. To indicate respiratory activity the presence of colour was determined after adding 10 µL/well of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (2 mg/mL) dissolved in water and incubation under appropriate cultivation conditions for 30 min

in the dark (Klančnik et al., 2010). All measurements of MIC values were repeated in triplicate.

Statistical analysis: The data were reported as mean values ± standard deviation (SD). Values representing the concentrations of investigated extracts that cause 50% neutralization or inhibition (IC_{50}) were determined by linear regression analysis of RSC (%) and LP inhibition results (Microsoft Excel program for Windows, v. 2007).

RESULTS AND DISCUSSION

The total phenolic content (Table 2) in examined oregano extract E1 is in accordance with previously published data (Chun et al., 2005). In postdistillation waste material extracts comparable values were obtained, and they are slightly higher then earlierly demonstrated (Albano and Miquel, 2011). Total flavonoid content in examined extracts was determined for the first time. E1 proved to have the highest concentration of flavonoids, followed by E4 (Table 2). Detailed chemical analysis showed that oregano extracts E1, E3 and E4 were abundant in rosmarinic, gallic and protocatechuic acid (Table 1) which is in correlation with previously published data (Chun et al., 2005). Also, flavonoids rutin, apigenin and quercetin were present in high amounts. The quantity of determined phenolcarboxylic acids and flavonoids does not vary significantly between normally produced and deodorised oregano herb extracts.

Table 1. The results of HPLC-DAD phenolic identification and quantification of investigated *Origanum vulgare* postdistillation waste extracts

Identified compound (mg/g d.e.)	**E1**	**E2**	**E3**	**E4**
Gallic acid	0.54	1.87	2.4	3.19
Protocatechuic acid	0.34	1.41	1.88	2.48
Caffeic acid	0.32	0.62	0.93	1
Chlorogenic acid	0.68	0.86	1.15	1.59
Syringic acid	0.04	0.47	0.68	0.92
Vanillic acid	0.11	0.44	0.59	0.85
Rosmarinic acid	**89.63**	**4.95**	**8.52**	**11.3**
Rutin	0.4	1.35	2.68	4.73
Naringenin	0.03	0.24	1	1.79
Quercetin	1.01	0.36	1.61	2.82
Apigenin	2.66	0.57	4.07	8.5

Antioxidant potential of investigated extracts partially follows the obtained results of chemical analysis. E1 possesses the strongest antioxidant activity in all investigated test systems. E3 and E4 are very similar in activity, whereas E3 is slightly more active. DPPH test on deodorised extracts was done by Albano and Miguel (2011), yet other assays regarding antioxidant capacity of oregano postdistillation waste material have not been performed. Interestingly, almost all samples were more active than synthetic antioxidant BHT in applied tests.

Table 2. Total phenolics (TP), total flavonoid (TF) and antioxidant activity (DPPH-RSC, OH-RSC and LP) of examined *Origanum vulgare* postdistillation waste extracts

Analyte	**TP** (mg GAE/g d.e.)	**TF** (mg QE/g d.e.)	**DPPH-RSC** IC_{50} (µg/ml)	**DPPH** AAI	**OH-RSC** IC_{50} (µg/ml)	**LP** IC_{50} (µg/ml)
E1	38.16	31.69	0.79	11.25	25.79	9.82
E2	25.16	18.13	2.74	3.24	41.55	25.78
E3	32.98	22.59	1.75	5.08	31.44	10.35
E4	36.2	29.31	2.14	4.15	33.71	12.89
BHT	/	/	**6.95**	**1.28**	**nr***	**14.71**

Antimicrobial activity of oregano herb on various bacterial strains has been well documented (Castilho et al., 2012). However, there is no data on antibacterial potential of postdistillation waste materials. All investigated extracts exhibited strong inhibition of growth of tested bacterial strains.

Table 3. Antimicrobial activity (expressed as MICs, in mg TP/mL) of oregano posdistillation waste extracts on investigated bacterial strains

Analyte	***Staphylococcus aureus*** **MIC** (mg TP/mL)	***Salmonella* Infantis MIC** (mg TP/mL)	***Escherichia coli*** **MIC** (mg TP/mL)	***Bacillus cereus*** **MIC** (mg TP/mL
E1	0.03	0.15	0.03	0.3
E2	0.06	0.18	0.13	0.13
E3	0.03	0.14	0.07	0.05
E4	0.05	0.10	0.05	0.02
BHT	0.55	0.55	0.55	0.28

E1, E3 and E4 were very similar in reached MIC values and all of them expressed a very high antimicrobial potential of oregano posdistillation waste extracts. Therefore, they should be further tested in food model studies for potential use as a relatively cheap source of effective natural preservatives in food industry.

CONCLUSIONS

In conclusion, results of this study confirmed *in vitro* antioxidant and antibacterial potential of ethanol extracts of oregano herb. High concentrations of rosmarinic acid, which has numerous pharmacological activities, such are antioxidative and antibacterial activities, were determined. Moreover, most of presented results, regarding chemical analysis, antioxidant and antibacterial activity of postdistillation plant waste material were obtained for the first time. Deodorised oregano herb extracts were found to be rich in active phenolic compounds and could be in the focus of future studies for isolation of caffeic acid oligomers or flavonoids. Also, their possible application in pharmaceutical and food industry should be considered.

ACKNOWLEDGEMENTS

The authors are thankful to ARRS, Slovenia and Serbia for financing of the bilateral project BI-034-SLO-SR-2010/11 and Regional Office for Science and Technological Development of Vojvodina (Grant No. 114-451- 205672011).

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RESISTANT STARCH AS FUNCTIONAL INGREDIENT IN HIGH-QUALITY FOOD

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ABSTRACT: Resistant starch (RS) from different sources has attracted huge interest, mainly because of its health benefits and functional properties. From technological aspect, positive advantage of RS is its lower impact on the sensory properties of food compared with traditional sources of fibre. The objective of this study was to observe functional properties of two types of RS (type 3 and type 4) as parts of dietary fibre, as well as, the possibility of its application in short dough cookies production.
Partial substitution of the flour with the RS (commercial sources) has contributed to significant changes in rheological and textural properties of the dough. In all samples the use of the RS resulted in the increase of storage and loss moduli of the dough, and the lowering of loss tangent, which indicates its more elastic character. Increasing the portion of RS, type 3 and type 4, as substitutes for flour, contributed to dough elasticity, due to the ability of RS to bind water.
The study involved baking test, as an important criterion for evaluating the quality of flour and RS. The application of RS in the formulation of short dough resulted in the products of improved sensory and nutritional quality. Addition of the starch, type 3, showed better results in terms of overall sensory quality. The findings show a good potential of the RS as a functional ingredient referring to its application in the production of cookies and related products rich in dietary fibre.
Key words: *resistant starch, dough, cookies, rheology, sensory properties*

INTRODUCTION

Recently, the use of starch in foods for health and well-being reasons has surged, resulting from the preponderance of perceived physiological benefits attributed to resistant starch (Sharma et al., 2008). As defined by Asp (1992), resistant starch is the „sum of starch and starch degradation products not absorbed in the small intestines of healthy individuals“. It is included in the definition of dietary fibre (Anon., 2001; Institute of Medicine, 2002). Four classes of resistant starch have been proposed (Sharma et al., 2008) on the basis of mechanisms of enzyme indigestibility, including inaccessibility of starch to amylases due to physical entrapment (RS1), inherent granular structure of raw starch (RS2), molecular association of amylose or retrogradation (RS3) and chemical modification (RS4). As discussed in a number of reviews (Nugent, 2005; Sajilata et al., 2006; Sharma et al., 2008; Fuentes-Zaragoza et al., 2010), resistant starch demonstrates the following physiological benefits in humans: modulation of blood glucose and insulin levels, positive effects on gastrointestinal health, increased absorption of minerals, prebiotic or bifidogenic effect, increased fat oxidation and fermentation into butyrate, which is touted to be protective against colorectal cancer.
Besides physiological benefits in human, RS has been reported to have potential as a unique ingredient that can yield high-quality foods (Yue and Waring, 1998; Baixauli et al., 2008a). RS has desirable physicochemical properties (Fausto et al., 1997) such as swelling, viscosity increase, gel formation and water-binding capacity, making it useful in a variety of foods. Application tests of RS show improved crispness and expansion in certain products and better mouthfeel, colour and flavour as compared with products produced with traditional, insoluble fibres (Sajilata et al., 2006). Applications of RS in foods have become a trend among food manufacturers to improve the health and well-being of consumers.

The objective of this study was to observe functional properties of two types of RS (type 3, type 4) as parts of dietary fibre, as well as, the possibility of its application in short dough cookies production.

MATERIALS AND METHODS

Materials

Flour ("Jaffa", Crvenka, Serbia) (composition data provided by the supplier: 13.9% moisture, 9.6% protein, 62.5% starch).
Resistant starch (type RS3) ActiStar C 11700 (CARGILL, Inc. Food and Pharma Specialties NA, USA). Moisture content was 12% and declared level of RS was 50.0%.
Resistant starch (type RS4) ActiStar RT 75330 (CARGILL, Inc. Food and Pharma Specialties NA, USA). Moisture content was 12% and declared level of total dietary fibre was 80.0%.
Seven formulations were prepared. One without addition of resistant starch (control) and six in which resistant starch (RS3 or RS4) was added as partial replacement of the flour in following concentrations: 5%, 10% and 15% (calculated on flour weight used in control sample). The formulations were prepared with 20% of water content in dough. Full recipes are given bellow in table 1.

Table 1. Full recipes of each formulation (given in presentage of each ingredient)

Ingredient	Control	Sample 1/4 (5% RS3/RS4)	Sample 2/5 (10% RS3/RS4)	Sample 3/6 (15% RS3/RS4)
Flour	55.6	52.8	50.0	47.3
Resistant starch	0	2.8	5.6	8.3
Sugar	19.4	19.4	19.4	19.4
Vegetable fat	11.7	11.7	11.7	11.7
NaCl	0.3	0.3	0.3	0.3
$NaHCO_3$	0.2	0.2	0.2	0.2
NH_4HCO_3	0.1	0.1	0.1	0.1
Distilled water	12.7	12.7	12.7	12.7

Viscoelastic properties of dough

Dough properties were determined after the three-hour relaxation using Haake Rheo Stress 600 (Karlsruhe, Germany). Elastic (storage) modulus (G') and viscous (loss) modulus (G'') were measured by a plate-plate sensor geometry at 20°C (60 mm in diameter with a 1-mm gap). Amplitude sweep was performed on 1Hz frequency, modulating shear stress values form 0-50 Pa in order to determine LVE regime. On the basis of determined LVE (linear viscoelastic) regime measuring for frequency sweep conditions were defined (Mezger, 2006). Dynamic oscillatory measurements (frequency sweep parameters) were observed modulating the ω value from 6.28-628 rad/s (frequency 1-10 Hz); in a constant value of shear stress (0.2 Pa). At least three replicates of each oscillatory shear test were conducted and good reproducibility was achieved since the differences between triplicates were less than 10%.

Baking test

Following the recipe and presentage of ingredients given in table 1 dough was prepared. Each ingredients was weighted in required quantities in order to form 350 g of dough. A required flour quantity was dosed in a z-mixer (Stephan-Werke Hamelin, ZD2245) and mixed for 0.5 min. Vegetable fat and sugar were added and mixing continued for 5.5 min (mixing speed 60 rev/min). Upon this resistant starch was added, mixed for 0.5 min, and then rising agents and distilled water were added. The mixer bowl was closed and dough was mixed during 15 min. Dough was shaped manually and placed into polyethylene bags. Dough relaxation time was 3 hours in the ultra thermostat at 20^{o}C. Before shaping the dough (50g)

was taken and viscosity measurement was carried out. Dough was pressed manually in a shape of a compact low cylinder. After 2 min of the relaxation time a dough stripe was developed by putting dough twice in either direction between two rollers of the laminator (Laminoir Marchand LA4-500). First lamination was with a 10-mm gap and second one was with 6-mm gap. After each lamination dough relaxed for 0.5 min. To avoid an adhesion a cloth stripe was floured by a small quantity of starch. After the relaxation time (2 min) dough was shaped by impressing a round mould (50 mm in diameter) on the surface of the dough stripe. Twelve pieces of shaped dough were moved to previously scaled perforated sheet-metal for baking, and again scaled to calculate a mean weight of shaped dough. Cookies baking time was 15 min at 230±2^{o}C. During baking the oven was closed and without the vapour connection. Cookies were cooled for 30 min on the baking sheet under ambient conditions.

Sensory evaluation

The sensory analysis of cookies was conducted by a six-member trained panel (Kamel and Stauffer, 1993). The sensory properties of cookies were determined by means of the sensory evaluation of the basic sensory parameters: external appearance, upper and bottom surface, snap, structure, mastication and taste (odor and taste). All seven sensory attributes were rated on a 1-5 scale where 1=lowest score and 5=highest score of each property.

Statistical analysis of data

Two batches of each dough formula were prepared on different days. Data reported for viscoelastic measurements was assessed by analyses of variance (ANOVA) and Duncan`s multiple test was used for any significant differences at the $P<0.05$ level between the means. All the analyses were conducted using statistical software package STATISTICA 8.1. (StatSoft Inc. USA).

RESULTS AND DISCUSSION

Viscoelastic properties of the dough are very important, as they influence the machinability of the dough as well as the quality of the final product. In all dough samples, regardless of the RS contents and the RS type, the elastic modulus G' dominated over the viscous modulus G" ($\tan \delta < 1$), indicating the prominence of elastic properties in the viscoelastic system (Figure 1). Because G' depended on angular frequency, and $\tan \delta > 0.1$ (Table 2), the observed rheological properties were typical for weak gels. Similar results were reported by Korus et al. (2009) in the gluten-free dough formulations with the addition of resistant starch (Hi-Maize 260 and ActiStar 11700).

Table 2. The values of tan δ of dough samples with resistant starch addition*

Sample	tan δ	
	RS3 addition	RS4 addition
Control	0.187 ± 0.001 a	0.187 ± 0.001 ab
5% RS	0.141 ± 0.015 c	0.190 ± 0.013 a
10% RS	0.134 ± 0.007 cd	0.166 ± 0.012 c
15% RS	0.184 ± 0.038 ab	0.155 ± 0.011 cd

** Different letters in the same column indicate statistical significance ($p < 0.05$).*

The supstitution of flour by RS3 or RS4 resulted in the increase of both dynamic moduli of dough compared to control sample once. The differences between control sample and samples with 5% and 10% of RS3 were statistically significant ($p=0.05$). The amount of RS3 of 15% significantly affected viscoelastic properties of the dough (G' of the sample was about 4 times higher than the control and other samples with the addition of RS3 and RS4).

Further, the differences between control sample and sample with 5% of RS4 were not statistically significant (p=0.05). The values of G' and G'' in the case of samples with 10% and 15% RS4 addition were statistically different than samples with 0% and 5% RS4 addition. Influence of the RS4 in the mechanical spectra of dough samples is not presented.

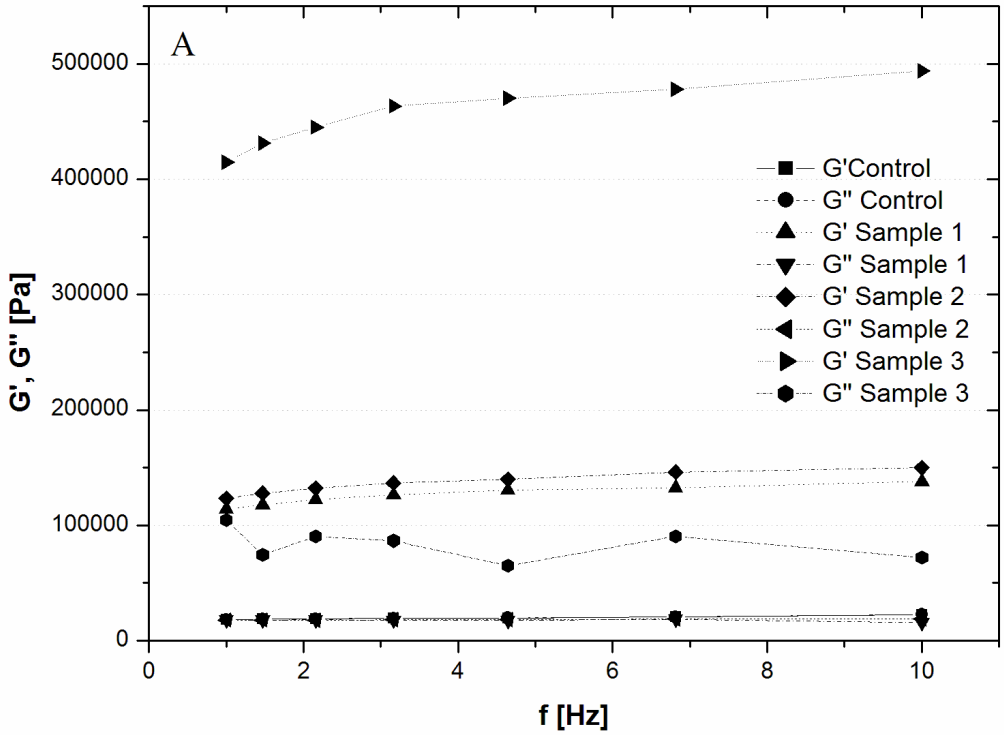

Figure 1. Influence of the RS3 in the mechanical spectra of dough samples at 20°C.

Increasing the portion of resistant starch, RS3 and RS4, as substitutes for flour, contributed to dough elasticity, due to the ability of resistant starch to bind water. With the rising level of resistant starch, water absorption of the dough is increased (the results are not given), and the addition of water has to be adjusted (in case of the highest dosage of RS3) in order to obtain proper consistency of the dough.

There are a numerous studies about partial replacement of flour with dietary fibres and the impact of the supplements on dough rheology and product quality. Most studies, however, referred to bread dough and breadmaking (Hung et al., 2007; Hung et al., 2005; Miyazaki et al., 2006). Hung et al. (2005) observed weakening of dough structure after addition of high amylose wheat flour. However, resistant starch (RS1 type) produced harder doughs for cookies production (Laguna et al., 2011). Differences in study results can be attributed to the existence of differences in physical and chemical characteristics of the applied sources of RS and studied rheological systems.

Sensory estimation of cookies

Sensory evaluation data (Table 3) shows that the RS3 addition in smaller quantities (5% and 10%) enhanced the appearance of the upper surface (the results are not given). The upper surface of cookies was smooth, slightly convex. The cookies structure was mealier and the change in odor and taste were detected (better than control). During mastication the tested samples dissolved well without mush making. The cookies with 5% of the RS3 had a desirable shape. The snap was distinctive; the structure was regular and homogeneous. The properties after mastication were adequate. The sample with 5% of RS3 addition was highly graded by 20 points after the sensory evaluation. With the increased RS3 content in the

formulations fat separated on the cookies surface. Beside that, the addition of 15% of RS3 in sample 3 resulted in the modification such as a denser and inadequate structure, which led to the decrease of total sensory quality compared to cookies without the RS3 addition.
The cookies with 5% RS4 addition had a regular shape and smooth upper surface. Its structure, snap and taste were identical to the control. During mastication the samples dissolved worse, while the odor was better than the control. The other two samples, with 10% and 15% of the RS4, had worse scores for features in mastication, odor and taste, resulted in decrease of the overall sensory quality compared to the samples with 0% and 5% of the RS4. Therefore, the partial substitution of the flour with the RS4 at the lowest dose (5%) gives the same total sensory quality as control sample.

Table 3. Sensory characteristics of short-dough cookies made from different blends of resistant starch and wheat flour*

Quality factors	Control	RS3 (%)				RS4 (%)		
		5	10	15		5	10	15
Appearance	5.0 a	5.0 a	5.0 a	4.3 c		4.0 d	4.5 b	4.0 d
Snap and structure	4.0 d	5.0 a	4.6 b	4.2 c		4.0 d	3.5 e	4.0 d
Mastication	4.6 b	5.0 a	5.0 a	4.0 c		3.5 d	3.5 d	3.5 d
Odor	4.0 c	5.0 a	5.0 a	4.5 b		5.0 a	3.5 d	3.0 e
Taste	4.5 b	5.0 a	5.0 a	4.5 b		4.5 b	3.5 c	3.0 d

** Values in a row followed by same letters do not differ significantly ($p < 0.05$).*

Formulating bakery products with resistant starches resulted in products with modified sensory properties (Baixauli et al., 2008b). However, these modifications did not affect the "overall acceptability" of these products to consumers. Sanz et al. (2008) also found that RS3 could be incorporated into the battered food up to a concentration of 20%, without comprising consumer acceptability.

CONCLUSION

Partial substitution of the flour with two types of RS has contributed to significant changes in rheological properties of short dough. By increasing the portion of RS (RS3, RS4) in the formulation of cookie dough, there was a significant increase in elasticity of the system, due to the ability of RS to bind water. The addition of 15% of the RS3 resulted in the modification such as an inadequate structure of the dough. In that case there is a need for modifications of the manufacturing process or the dough formula. Application of the resistant starches in the formulation of short dough resulted in a product of improved sensory and nutritional quality. Better results in terms of overall sensory quality were obtained by applying the starch type RS3. The results show good potential of resistant starch as a functional ingredient in terms of its application in the production of cookies and related products rich in dietary fiber.

ACKNOWLEDGEMENTS

This study was financially supported by the Ministry of Education and Science of Republic of Serbia, Project TR31069.

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NUTRITIONAL QUALITY OF LINSEED AND OIL HEMP VARIETIES

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ABSTRACT: Oil hemp and linseed can be a complete and balanced source of fatty acids. Oil hemp has an optimal ω-6 / ω-3 ratio of 3:1 (Callaway, 2004), but with limited use in Romania because of the stigma of drug, and linseed, due to the oil quality which contains ω-3 and ω-9 fatty acids and the pleasant taste, in addition to traditional uses, increasingly finds new uses in areas where until recently there were no data on the use of those cultures: food industry (pastry/bakery), medicine and cosmetics. The biological material used consisted of two monoecious hemp varieties (Zenit and Denise), one dioecious hemp variety (Armanca) and three flax oil varieties (Lirina, Florinda and Iunia 96) authorized in Romania. The oil content of the studied varieties was determined by Soxhlet method with a Velp block of mineralization. Investigation of fatty acid profile was performed by gas chromatography GC-MS whit Shimadzu GC MS QP 2010. The compounds separated and identified from the hemp and linseed oil were: linoleic acid ω-6, alpha-linolenic acid ω-3, oleic acid ω-9, palmitic acid, stearic acid. Due to the fatty acid profile, linseed and oil hemp are highly suitable dicotyledonous crops for the human health.
Key words: *fatty acid, oil hemp, linseed*

INTRODUCTION

The great economic value of hemp and linseed is defined by the multiple possibilities of use. Hemp can be used for fiber, seeds and oil, wood, manure and chaff in medicine by hypnotic and psychotropic alkaloids blossom while linseed is used in the varnish, paints and printing inks industry (Tabără, 2005), medicine, food industry and cosmetics.
Hemp seeds (*Cannabis sativa* L.) are distinguished by their complex and original composition of fatty acids, proteins, vitamins and minerals they contain. Whole hemp seeds have oil content of about 25-35 % (Şandru et al., 1996), but in Russia there is a cultivated variety called "olifera" which contains 40 % oil (Deferne et al., 1996). About 30–35 % of the weight of hemp seed is an edible oil that contains about 80 % essential fatty acids (EFAs): linoleic acid ω-6 (LA, 55 %), alpha-linolenic acid ω-3 (ALA, 22 %), in addition to gamma-linolenic acid ω-6 (GLA, 1–4 %) and stearidonic acid ω-3 (SDA, 0–2 %).
Hemp oil is valued primarily for its nutrients and for health benefits, because of the content of saturated fatty acids (myristic acid, stearic, palmitic, eicosanoic), especially rich unsaturated fatty acid (oleic acid, alpha-linoleic acid). The major components of hemp oil are triglycerides together with sterols, tocopherols, phospholipids, terpenes and so on, all of which have a beneficial effect on the body.
Hemp seeds not only contain essential fatty acids, but come with substantial contribution of 20-25 % protein, essential amino acids, which make hemp seed an ideal food for vegetarians, successfully replacing the lack of animal protein, with a high nutritional value for human consumption in salads, bread and even chocolate.
On the other hand, cold pressed linseed (*Linum usitatissimum* L.) oil is 100 % natural. It has special qualities, containing two times more ω-3 fatty acids than fish oil and a mixture of essential ω-3 and ω-9 fatty acids. All these amino acids have a role in cell recovery, being very useful in various diseases. Protein from linseed oil is easily digestible and contains all the amino acids necessary to maintain a strong and healthy body. Linseed oil has positive

effects on blood pressure, lower cholesterol, constipation, psoriasis, eczema, burns and a role in preventing heart attacks.
Flax seeds contain oil which has in its composition 55 % ω-3 fatty acids in a ratio of 2/1 from ω-6. Flaxseed germs contain: ω-3 fatty acids; lignite, pectin, linamarine; 35 % vegetable oils, of which 75 % essential fatty acids; mucilage; vitamins (A, B, C, D and E); minerals. Flax seeds have very important fiber content. One cup (50 g) of flaxseed contains 20 grams of fiber.
The aim of the study presented in this paper was to investigate fatty acid composition in hemp oil and linseed, and to obtain overall picture about their nutritional suitability, from the point of fatty acids.

MATERIAL AND METHODS

The analyzed samples consisted of two monoecious hemp varieties (Zenit and Denise), one dioecious hemp variety (Armanca) and three flax oil varieties (Lirina, Florinda and Iunia 96) authorized in our country, according to the Official Catalogue of varieties of crop plants in Romania. The oil content of the studied varieties was determined by Soxhlet method with a Velp block of mineralization. Investigation of fatty acid profile was performed by gas chromatography GC-MS whit Shimadzu GC MS QP 2010.

Oil content determination

Soxhlet method with a Velp block of mineralization was used for oil content determination. This method is a lengthy process requiring up to a day for a single analysis. The solvent extraction step alone takes six hours. The fat content was determined by extracting the fat from the samples using a solvent (petroleum ether), and after that the weight of the fat recovered was determined. The sample is contained in a porous thimble that allows the solvent to completely cover the sample. The thimble is contained in an extraction apparatus (Figure 1) that enables the solvent to be recycled over and over again. This extends the contact time between the solvent and the sample and allows it time to dissolve all of the fat contained in the sample. In order for the solvent to thoroughly penetrate the sample it is necessary for the sample to be as finely comminuted as possible. Necessary equipment consists of analytical balance (at least 1 mg sensitivity), electrical drying oven operated at 102 ºC ± 1 ºC, Soxhlet extraction unit (150 ml round bottom flask, Soxhlet extractor with 60 ml siphoning capacity and condenser, 28 x 80 mm cellulose extraction thimbles), fume cupboard, heat source, desiccator with silica gel desiccant, glass rod. Oil content is determined by the formula: % crude fat = (W2 – W1) x 100/S

W1 = weight of empty flask (g)
W2 = weight of flask and extracted fat (g)
S = weight of sample

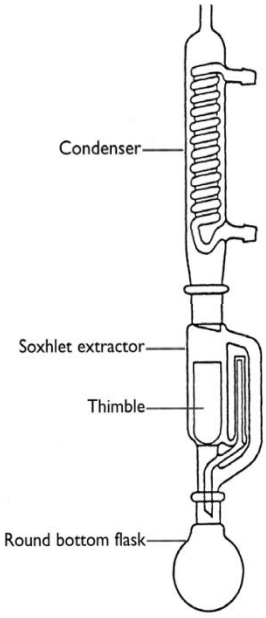

Figure 1 – Soxhlet extraction apparatus

Fatty acid determination

Fatty acids were determined by gas chromatography. Lipids were extracted following the Folch method. Fatty acid profiles of samples were separated and identified by using a GCMS-QP 2010+SHIMAGZU gas chromatograph, equipped with a AT-5MS (30 m x 0.32 mm inside diameter) capillary column of silica. The oven program was the following: 70 ^{0}C for 2 minutes, than it was heated to 150 ^{0}C with a gradient of 10 ^{0}C / minute and than, a floor of 3 minutes, after that it was raised again to 235 ^{0}C with a gradient of 4 ^{0}C / minute. The temperature of the injector was 260 ^{0}C, injection mode split, split ratio 1:20. Helium was used as carrier gas (Fota et al., 2010).

RESULTS AND DISCUSSION

The results of fatty acid analysis made from the oil obtained by extraction with petroleum ether (Figure 2), are shown in Table 1.

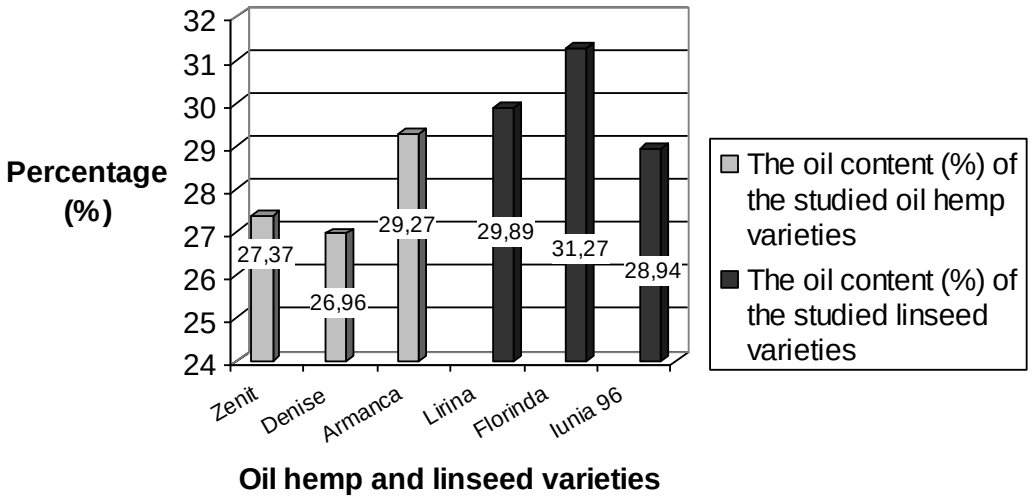

Figure 2 – The oil content (%) of the studied oil hemp and linseed varieties

As it can be seen from the results, most prevalent fatty acids in oil hemp are 18:2 ω-6 (linoleic acid) and 18:3 ω-3 (alpha-linolenic acid). Instead, linseed has a higher concentration of 18:3 ω-3 (alpha-linolenic acid) and 18:1 ω-9 (oleic acid) fatty acids. These results are expected and in consistence with other literature data (Sauvant et al., 2004).
Saturated fatty acids (SFAs) have been generally labeled as the cause of cancers and coronary heart disease. The mean ratio of polyunsaturated fatty acids (PUFA) / saturated fatty acids (SFA) recommended by the FAO experts is above 0.4 (Wood et al., 2008). From this point of view, of saturated fatty acids concentration, all examined samples have had favorable values between 2.9 and 7.1 in oil hemp and between 2.82 and 5.5 in linseed (figure 3).

Table 1. Fatty acid composition (% w/w) of oil hemp and linseed

Variety / Fatty acid	OIL HEMP			LINSEED		
	Zenit	Denise	Armanca	Lirina	Florinda	Iunia 96
Palmitic acid (16:0)	7,10	6,64	6,75	5,50	5,40	4,65
Stearic acid (18:0)	2,90	2,68	1,21	3,32	4,92	2,82
Oleic acid (18:1) ω-9	13,97	14,02	8,58	21,73	19,95	27,40
Linoleic acid (18:2) ω-6	57,57	57,80	64,79	17,26	15,40	14,39
Alpha-linolenic acid (18:3) ω-3	14,98	15,85	18,67	52,17	54,30	50,71

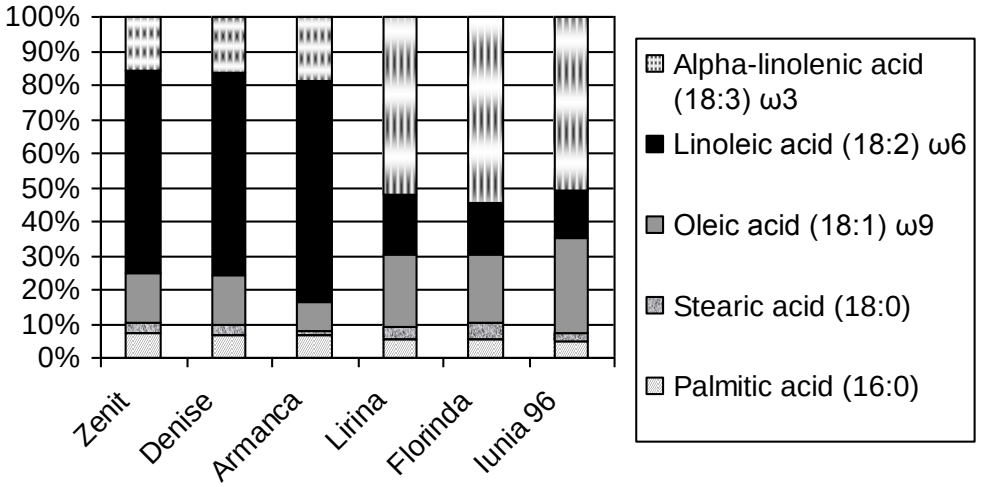

Figure 3 – Oil hemp and linseed fatty acid composition in examined samples (% w/w)

The optimum ratio between monounsaturated, polyunsaturated and saturated fatty acids it has to be 1:1:1. Equally important is the ratio between the essential fatty acids ω-6 and ω-3. Between the two types of polyunsaturated fatty acids there must be a relationship of the following type: ω-6/ω-3 = 5:1 (ω-6 = 5 g/day, ω-3 = 1 g/day).
It has been estimated that the present Western diet is deficient in ω-3 fatty acids, with a ratio of ω-6 to ω-3 of 15-20/1. It is therefore recommended increased consumption of foods rich in ω-3 fatty acids like fish (trout, salmon) or linseed oil.
The results obtained from the analyzed samples show the optimal ω-6 / ω-3 ratio of fatty acids in hemp oil and the appropriate mixture of essential ω-3 and ω-9 fatty acids in linseed. Therefore, both linseed and oil hemp can be used in human nutrition as a balanced source of essential fatty acids, with a plus for linseed.

CONCLUSIONS

If we analyse the fatty acid composition of the studied oil hemp and linseed varieties, we can say that they are suitable for food and feed usage. Linseed varieties have had a concentration of ω–3 fatty acid between 50.71 and 54.3 and low saturated fatty acids values that make it one of the essential foods for a balanced diet. On the other side, oil hemp has lower levels of ω–3 fatty acids but compensates with higher ω–6 levels and, like linseed, it has low concentrations of SFAs. Knowing this, we can say that linseed and oil hemp are highly suitable dicotyledonous crops for the human health.

ACKNOWLEDGEMENTS

The results of this study are part of a PhD program, having as theme: "The contribution of some ecological and technological factors in quality formation of the production in flax oil" and "The contribution of some ecological, biological and technological factors in quality of the hemp seed oil", funded by the European Social Fund, the Pilot Program PhD Research Scholars Support Contract from the POSDRU/CPP107/DMI 1.5/S/80127.

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THE IMPORTANCE OF FUNCTIONAL FOOD IN HUMAN NUTRITION AND COMPARISON WITH CONVENTIONAL PRODUCTS

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ABSTRACT: The functional food is a modern concept in human nutrition, considering the fact that this type of food contains ingredients which have useful effects on one or more purpose functions in the organism and contribute to the general medical health and/or decrease the risk of some diseases. The ingredients of functional food are: macronutritients with special physiological treatment (polysaccharides, omega-3 fatty acids, etc.), essential micronutritients (folic acid, vitamin B6, B12), nonessential ingredients (some of oligosaccharides), biologically active phytonutritients (carotenoides, flavons, catechins, etc.), friendly bacteria – probiotics. Modern research indicates expansive development and production of functional food around the world. That requires appropriate legislation which defines the field of functional food production. The objectives of this study are to emphasize the importance of functional food in daily nutrition. Furthermore, using sensory analysis for three different product types with functional characteristics, it was proved that the food with functional characteristics does not differ in total values from the similar conventional products.
Key words*: functional food, omega-3, omega-6, bioactive substances, conventional products*

INTRODUCTION

While food has been used to improve health for a long time, our knowledge of the relationship between food components and health is only at the outbreak to improve food. Roughly speaking, all food is functional if it provides energy and nutrients necessary for survival. But the term “functional food” that is in use today conveys health benefits that extend far beyond mere survival. Food and nutrition science has moved from identifying and correcting nutritional deficiencies to designing foods that promote optimal health and reduce the risk of disease (IFT Expert Report, 2005). According to the definition functional food is part of everyday diet and is demonstrated to offer health benefits and to reduce the risk of chronic disease beyond the widely accepted nutritional effects (Hasler, 1998). The concept of functional foods was developed in Japan in the 1980s, with a formal definition of “foods for specified health use”(FOSHU) in 1991, accompanied by a regulatory system to approve the statements made on labels and in advertising, based on scientific evaluation of the evidence of efficacy and safety (Shimizu, 2003). The functional food comprises of: conventional foods containing naturally occurring bioactive substances (e.g., dietary fibre), food enriched with bioactive substances (e.g., probiotics, antioxidants), synthesized food ingredients introduced to traditional foods (e.g., prebiotics) (Raghuveer and Tandon, 2009).
Breadbury et al. (1996) have mentioned that adding of vitamins and minerals especially in the breakfast cereals, is an established concept. Following that trend, the range of food product which contain added vitamins and minerals has been increasing, as well as introduction of different antioxidants, vitamins A, C, E and beta-carotene in composition of

the varius products. They also reported the existing bond between consumption of oily fishes, which are rich in omega-3 fatty acids and the reducing rates of coronary heart desease. Therefore, those are added to margarine, biscuits, bread and cakes. This is supported by the health claims, which are made on the basis of the fact that the consumption of these products could reduce the risk of coronary heart disease. Scientific evidence, based on controlled intervention trials in humans, suggested dietary omega-6/omega-3 fatty acids recommendations depending on specific physiological situations (adults with different pathologies, infants, newborns, etc.) (Simopoulos, 2002). A frequently used definition of probiotics is "living food supplements or components of bacteria that have been shown to have beneficial effects on human health" (Sheil et al., 2007; Weichselbaum, 2009). The most common type of probiotic functional foods are dairy products such as yoghurts and yoghurt/dairy drinks which have been modified by the addition of a particular strain of live microorganism in the form of lactobacteria such as *Lactobacillus* and *Bifidobacterium* and in some cases prebiotics such as inulin as well. The additional health benefits claimed by probiotic dairy products are related to the gastrointestinal functions and include improving digestion and immunity and managing digestive disorders such as IBS (irritable bowel syndrome) and diarrhoea (ILC-UK, 2010). Soymilk is alternative source of the soy protein, which can replace the dairy animal milk in everyday consumption and it is present on the market in different flavours, including chocolate, vanilla, plain etc. Also it can be used as dairy milk substitute in coffee, tea or cereals (Hajirostamloo, 2009). A number of dietary agents, including soluble fibres and plant sterols/stanols, were found to interfere with cholesterol absorption and to lower its levels in serum. Plant sterols and stanols, also called phytosterols and phytostanols, have chemical structures resembling that of cholesterol but are only available to humans through plant foods such as vegetable oils, nuts, seeds, cereals, legumes, fruits, and vegetables or industrial supplements from plant origin (Piironen et al., 2000).

The addition of probiotic bacteria in yoghurt is made not only because of certain claimed health-promoting effects in the intestinal tract. Other beneficial health effects suggested to include the enhancement of the immune system, reduction of lactose intolerance, control of diarrhea and reduction of LDL cholesterol (so called "bad cholesterol") (Scheinbach, 1999). Soy milk seems to be a healthier choices because it is low in calories, carbohydrate, fat and fatty acids and is important for people who are allergic to cow milk protein and lactose (Hajirostamloo, 2009). Besides being a suitable options for the lactose intolerants (Potter et al., 2007), soy-based desserts mixed with fruit juices are a new trend of products that include soy-protein in the regular diet (Granato et al., 2010). Also new trend for consumption of healthy, innovative and practical food, which has occurred recently, has leaded the market of cereal-bars to a gradual growth. Cereal-bars are considered healthy type of food, because they are rich in fiber, however, poor in fat (Bower and Whitten, 2000; Palazzolo, 2003; Gomes and Montenegro, 2006).

All the nations have a different regulatory framework for functional food. Although each country is in process of creating adequate regulatory structure for the functional foods but there are certain challenges ahead for the regulatory bodies, food scientists, manufacturers and even consumers which needs to be addressed for the success of functional foods (Kaushik and Kaushik, 2010).

From a marketing perspective, it is important to understand that consumers weigh different factors when making a purchase decision and these include not only health and nutrition but also price, convenience, and taste (Verbeke, 2008). Taste, in particular, has been regarded as the critical factor for functional food's acceptance, along with trustworthiness of health claims—consumers will rarely compromise on taste for foods with potential health benefits (Verbeke, 2006; Urala and Lähteenmäki, 2004).

The aim of this study was to evaluate using sensory analysis various types of functional products in comparison with conventional products. The foods that were sensory analyzed were probiotics in compare with natural yoghurts, chocolate pudding with chocolate soya dairy-free dessert and functional food cereal bars with conventional bars.

MATERIAL AND METHODS

Three different brands of probiotics (Viva, AB and Balans +) and three conventional yoghurt brands (Dukat, Family and Jogurt), Alpro chocolate soya dessert enriched with calcium and vitamins B2, B12 and D2 along with three different manufacturer chocolate puddings (Dukat, Campina and Zott), three types of functional cereal bars (Mond, KitKat and Albeni) and three various brands of conventional sweets (Cerbona, Bonžita and Vitanova), were purchased from mega markets and retails in Novi Sad.
The method which is used in this research was descriptive rating test. The appropriate attributes for the evaluation of yoghurts and puddings were appearance, odor, flavor/taste, texture and aftertaste. In case of cereal bars, categories were appearance, flavor, audition (crunchiness) and texture. During the quantitative assessment, 3 panelists have evaluated samples using a numeral ordinal 5-unit scale. Every unit is described by certain descriptors, which are discussed and agreed upon the definitions by the panelists, with the end values labeled as not appropriate or imperceptible (1) and all rounded or satisfactory (5). Before the sensory evaluation all the samples were prepared in the same conditions. The yoghurts were in identical transparent glass cups, the puddings were served in plastic cups, and the cereal bars were without wrapper. Every sample got his personal digit code.

RESULTS AND DISCUSSION

Individually and summary ratings, by upon discussed attributes, of probiotic and conventional yoghurts are shown in Table 1.

Table 1.. Sensory evaluation of functional and conventional yoghurt

Categories	Brands						Summary	
	VIVA (P)	Dukat	AB (P)	Family	Balans + (P)	Jogurt	Probiotics	Conven-tionals
Appearance	4.00	3.67	4.67	3.67	3.33	4.67	4.00	4.00
Odor	4.33	4.67	4.67	4.00	4.00	3.67	4.33	4.11
Flavor	3.67	3.33	4.00	4.00	3.33	4.00	3.67	3.78
Texture	3.67	3.67	4.67	4.00	4.33	4.67	4.22	4.11
Aftertaste	4.33	3.33	4.33	4.33	4.00	4.00	4.22	3.89
Average score	4±0.76	3.73±1.03	4.47±0.64	4±0.85	3.8±1.08	4.2±0.86	4.09±0.87	3.98±0.92

The average values in total of probiotic and conventional yogurt were 4,09 and 3,98 with a standard deviation of 0.87 and 0.92 respectively. The noticeably higher sensory quality of the sample “AB Subotica“, containing probiotic cultures *Lactobacillus acidofilus* LA-5 and *Bifidobacterium* BB-12 and Inulin as prebiotic, which could be attributed to certain different properties of texture and appearance. In general, differences between probiotics and conventional yoghurts are minor, with marginally higher organoleptic quality in most categories for probiotics, beside the flavor, which rate is slightly higher in case of conventional yoghurts (Figure 1).
The results of the sensory evaluation of chocolate puddings and chocolate soya dessert are represented in Table 2.

Table 2.. Sensory evaluation of chocolate pudings and soya dairy-free dessert

Categories	Brands				Summary	
	Dukat	Campina	Zott	Alpro soya (F)	Alpro soya (Functional)	Conventionals
Appearance	4.33	4.33	3.67	4.00	4.00	4.11
Odor	4.00	4.33	4.00	3.67	3.67	4.11
Flavor	4.00	4.33	3.00	4.00	4.00	3.78
Texture	4.33	4.33	3.67	4.00	4.00	4.11
Aftertaste	4.33	4.33	3.33	4.33	4.33	4.00
Average score	4.2±0.86	4.33±0.49	3.53±0.83	4±0.76	4±0.76	4.02±0.81

From the table 2 it is obvious that the average value for the functional dessert is 4, while the average score for the conventional products is almost the same (4.02). Standard deviations for soy dessert and conventional puddings are 0.76 and 0,81, respectively. The best overall result is obtained for Campina conventional pudding, with very good taste and better odor then other samples. In summary, soya dessert is better in flavor and aftertaste category in compare with conventional puddings. Deficiency for this functional dessert is insufficiently original pudding odor (Figure 2).

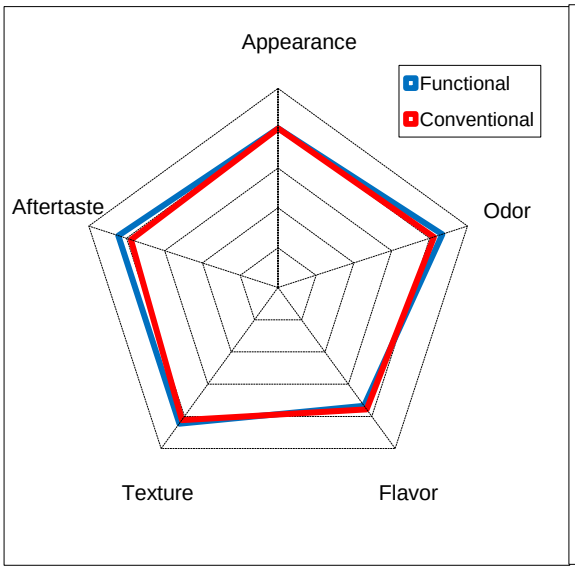

Fig. 1. Ratings by categories for yoghurts

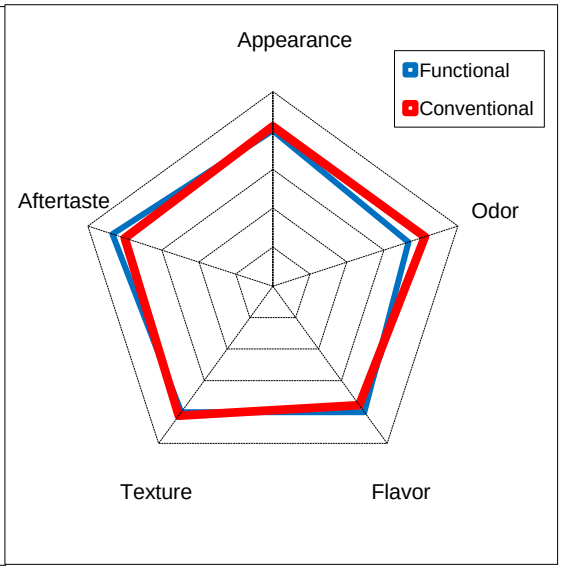

Fig. 2. Ratings by categories for puddings

Table 3 shows average values of sensory analysis for the functional cereal bars and the conventional sweets.

Table 3.. Sensory evaluation of functional and conventional cereal bars

Categories	Brands						Summary	
	Mond	Cer-bona(F)	KitKat	Bonžita (F)	Albeni	Vita-nova(F)	Functio-nals	Conven-tionals
Appea-rance	4.67	4.33	4.67	4.33	4.33	4.33	4.33	4.56
Audition	3.67	4.67	5.00	4.33	2.67	4.67	4.56	3.78
Flavor	4.00	3.67	4.33	4.67	3.67	4.67	4.33	4.00
Texture	4.33	5.00	4.33	4.67	3.67	4.67	4.78	4.11
Average score	4.17±0.94	4.4±0.67	4.58±0.51	4.5±0.52	3.58±1	4.58±0.67	4.50±0.61	4.11±0.92

According to the values from table 3, the functional cereal bars have higher average ratings compared to the conventional bars (4.5±0.61 vs. 4.11±0.92). The conventional treat KitKat (Nestle) is evaluated with the best score from the category of non-functional bars, especially concerning crunchiness. Vitanova macrobiotic with dietary fibers from Pionir Subotica has exactly the same average value like KitKat and this product is the leader in the functional bars category, characterized by the very good audition, flavor and texture. The weakest point of the functional cereal bars was the appearance and the best characteristic was homogeneous structure. Lack of chewiness and crunchiness were the major disadvantages of the conventional sweets (Figure 3).

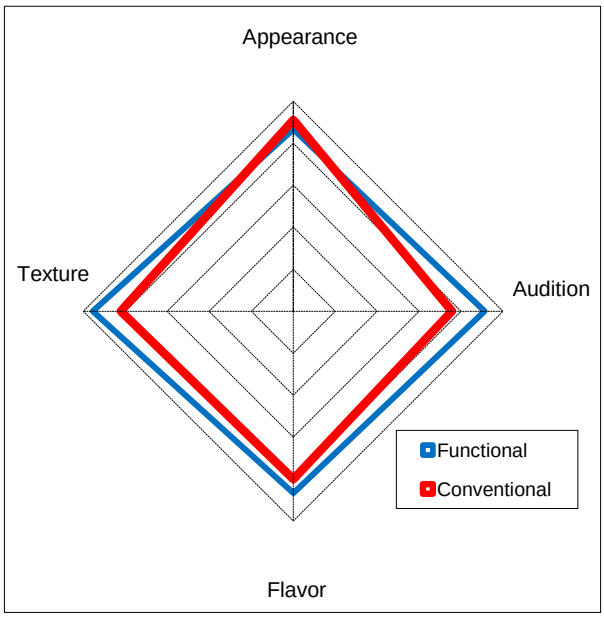

Fig. 3. Ratings by categories for cereal/conventional bars

CONCLUSIONS

For the reason of nonregulated legislation framework in many countries, trust in the certain health claims is still optional for individuals, also as consuming of functional food. Still, there are strong evidences that functional food can reduce the risk of chronic disease. As mentioned earlier, some prejudices about taste of the functional food reject certain group of consumers. Appearance, flavor and texture are the important factors, which determine the acceptance or rejection of a food article. According to this investigation, differences in sensory quality of some functional and conventional groceries are minimal. Conventional products with ingredients which may lead to detrimental long term health effects, can easily be replaced in daily nutrition with functional food of the same category. That means that

consumers can affect their general medical health with slightly modifying their nutrition habits. Also, consumers must be aware that functional food is not a magic bullet that works alone, but may promote good health when is included as part of a healthful diet, regular exercise, stress reduction and healthy body weight. The ultimate goal may be reduction of overall medical expenses for treatments and achieving a healthier nation.

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THE QUALITY OF A GLUTEN-FREE DIET EVALUATED WITH MEDITERRANEAN DIETARY QUALITY INDEX IN CROATIAN CELIAC PATIENTS

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ABSTRACT: Strict gluten-free diet, the only treatment for patients with celiac disease, is necessary to prevent malabsorption and to reduce the risk of complications. Such diet excludes all food and beverages that contain gluten and therefore may result in a nutritionally unbalanced diet. Mediterranean diet is a synonym for healthy and balanced diet. It has been shown that greater adherence to Mediterranean diet is correlated with reduced mortality. Adherence to Mediterranean diet can easily be determined using Mediterranean dietary quality index (MDQI). MDQI is consisted of seven components (saturated fatty acid, cholesterol, meats, olive oil, fish, cereals, fruits + vegetables), whereas each component depending on intake may achieve 0 to 2 points. Total index score might be between 0 and 14 points, where 0 points indicate great adherence to Mediterranean diet. The aim of this study was to evaluate the quality of a gluten-free diet based on a MDQI in Croatian celiac patients. MDQI was calculated based on a seven-day food record from 23 celiac patients from Mediterranean region and 38 celiac patients from continental region. Statistical difference was observed only for saturated fatty acid intake among observed regions ($p<0.05$). Celiac patients from Mediterranean region achieved 9.79 points while celiac patients from continental region achieved 10.68 points, however there is no statistical significant difference between these regions. In conclusion, celiac patients adhering gluten-free diet, probably due to specific dietary restriction, as expected have lower diet quality according to MDQI.
Key words: *diet quality, gluten-free diet, Mediterranean diet*

INTRODUCTION

Strict gluten-free diet is the only currently available treatment for patients with celiac disease. It is necessary to prevent malabsorption and it will results in symptomatic, serologic and histological remission in the majority of patients (Pietzak, 2005). Gluten-free diet excludes all food and beverages that contain gluten, a protein found in wheat, rye, barley and oats. Previous research showed that gluten-free diet may result in a nutritionally unbalanced diet (Grehn el al., 2001; Mariani et al., 1998; Hopman et al., 2006). Mediterranean diet has been promoted as a model for healthy and balanced diet. Adherence to Mediterranean diet is correlated with reduction in cardiovascular diseases and cancer mortality (Trichopoulou et al., 2003; Knoops et al., 2004; Dontas et al., 2007). Although, there is no single Mediterranean diet (Noah and Truswell, 2001), Mediterranean diet pattern is characterized by a high intake of vegetables, fruits, legumes, cereals, olive oil, low consumption of meat and moderate alcohol consumption (Trichopoulou et al., 1995). Adherence to Mediterranean diet pattern can easily be determined using a Mediterranean dietary quality index (MDQI) (Gerber, 2006). The aim of this study was to evaluate the quality of a gluten-free diet using a MDQI in Croatian celiac patients.

SUBJECTS AND METHODS

The survey was carried out on a 61 celiac patients, aged 18 to 69 years, from two different regions in Croatia: Mediterranean (n=23) and continental region (n=38). All patients had biopsy proven celiac disease and were adhering gluten-free diet in average 9 years.

Subjects compiled a 7-day food record for which they were instructed by trained dietitians to record completely all food and drinks consumed in observed period. Subjects were provided with forms that were designed to collect information such as food item, brand, type and preparation method. They were asked to weigh food and to use cups or tablespoons for estimation when weighting was not possible. The intake of energy and nutrients were calculated using national food composition table (Kaić-Rak i Antonić, 1990). Patients were asked to provide food labels of all gluten-free products consumed in that period because these products are not included in food composition table.
Mediterranean dietary quality index was calculated based on 7-day food records. Scores 0, 1 and 2 were assigned to each of the seven component (saturated fatty acid, cholesterol, meats, olive oil, fish, cereals, fruits + vegetables) depending on achieved intake (Gerber, 2001). The meat group included all processed and fresh meat; fish included white and fatty fish; the cereals group included all types of bread, pasta, rice and breakfast cereals; the fruit and vegetables group consisted of cooked and raw types of red, yellow and green vegetables and fresh fruit (Scali et al., 2000). Scores were summed for each subject, giving the total score for the MDQI. Results are presented as mean ± SD, or percentage. Differences between regions were assessed using Student's t-test. All statistical analysis was performed with SPSS software v. 15.

RESULTS AND DISCUSSION

In the present study, MDQI was used to evaluate the quality of the gluten-free diet in Croatian celiac patients from two different regions (continental and Mediterranean). Subjects from both regions consumed too much saturated fat (>10 % kJ) and meat and insufficient amount of cereals, fruits and vegetables, olive oil and fish (Table 1). High intake of saturated fat was also observed among healthy Croatian population (Primorac et al., 2003). High consumption of red meat was found among Croatian population in the first Croatian Health Project conducted in 1995-1997 (Turek et al., 2001). Low intake of cereals may be due to special restriction in the gluten-free diet. Adherence to gluten-free diet primarily affects consumption of cereals. Mariani et al.,1998 found higher energy intake from fat and lower energy intake from carbohydrates in adolescents celiac patients on a gluten-free diet. All fruits and vegetables are allowed in the gluten-free diet. However, 89% of subjects consumed less then 400 g fruits and vegetables per day. Individuals who consume very low amounts of fruits and vegetables have the greatest risk of colorectal cancer (Terry et al., 2001). Low consumption of fish among subjects from Mediterranean region was very surprising. Pucerin Cvetković et al., (2006) found that 35% of Croatian island population consume fish 3 to 4 times a week while 9.8% consume 5 to 7 times a week. Very low consumption of olive oil (< 5 mL/day) was observed in both regions. Although Croatia is a country in the Mediterranean region the consumption of olive oil is less than 1L per capita per year unlike some countries from Mediterranen region such as Greece, Italy and Spain where consumption exceeds 10 L per capita per year (Grigg, 2001). High intake of olive oil, because of its high levels of monounsaturated fatty acids and polyphenolic compounds, have a role in a prevention of cardiovascular disease and cancer (Owen et al., 2004).
Since the MDQI was calculated based on seven day food record it is possible that some of the diet pattern are underestimated due to recording period. An individual's nutrient intake may fluctuate on a daily basis and season of the year (National Research Council, 1986).

Table 1. Average daily intake of components included in MDQI based on seven day food record in celiac patients (n=61)

Component	Mediterranean region (n=23)	Continental region (n=38)	p
Saturated fatty acid (% kJ)	12.41 ± 3.22	15.17 ± 3.27	0.005*
Cholesterol (mg)	341.44 ± 290.68	329.86 ± 148.16	0.848
Meats (g)	179.84 ± 121.44	150.91 ± 94.30	0.339
Olive oil (mL)	2.24 ± 3.08	1.20 ± 2.65	0.201
Fish (g)	23.52 ± 28.72	13.30 ± 19.86	0.133
Cereals (g)	186.68 ± 152.96	172.01 ± 77.17	0.644
Fruits and vegetables (g)	276.96 ± 147.86	242.80 ± 169.81	0.466

*Results are given as mean ± standard deviation, *p<0.05*

The average score for each component included in the MDQI is shown in Table 2. Difference between regions was observed only for saturated fatty acid. Subjects from continental region had statistically higher intake of saturated fatty acid than subjects from Mediterranean region ($p<0.05$) (Table 1). The proportion of energy intake provided by saturated fat was over 10 % in 97% of the subjects from continental region. Subjects from Mediterranean region had lower total MDQI score (9.79) than the subjects from continental region (10.68), however no statistically significant difference was observed ($p=0.083$) (Table 2). Although some authors have found the difference in dietary pattern among different regions in Croatia (Doko Jelinić et al., 2009), according to Šatalić et al., (2004) Croatian students have lower diet quality evaluated with MDQI, with no significant difference between the regions.

Table 2. Average score of components included in the Mediterranean dietary quality index calculated from seven day food record provided by celiac patients adhering gluten-free diet (n= 61)

Component	Score		p
	Mediterranean region (n=23)	Continental region (n=38)	
Saturated fatty acid (% kJ)	1.32 ± 0.75	1.74 ± 0.57	0.042*
Cholesterol (mg)	0.53 ± 0.70	0.82 ± 0.87	0.207
Meats (g)	1.58 ± 0.51	1.50 ± 0.51	0.589
Olive oil (mL)	1.84 ± 0.37	1.91 ± 0.29	0.452
Fish (g)	1.63 ± 0.60	1.74 ± 0.57	0.534
Cereals (g)	1.05 ± 0.62	1.12 ± 0.54	0.691
Fruits and vegetables (g)	1.84 ± 0.50	1.85 ± 0.44	0.935
Total MDQI score	9.79 ± 1.81	10.68 ± 1.72	0.083

*Results are given as mean ± standard deviation, *p<0.05*

No subject scored between 0 and 4 (Figure 1). Only 5% of subject from Mediterranean region had score 5, which may be categorized under “medium-to-good MDQI”. The majority of subjects (68%) from Mediterranean region had “medium-to-poor MDQI” (5 to 10), while the majority of subjects from continental region (53%) had “poor MDQI” (11 to 14).

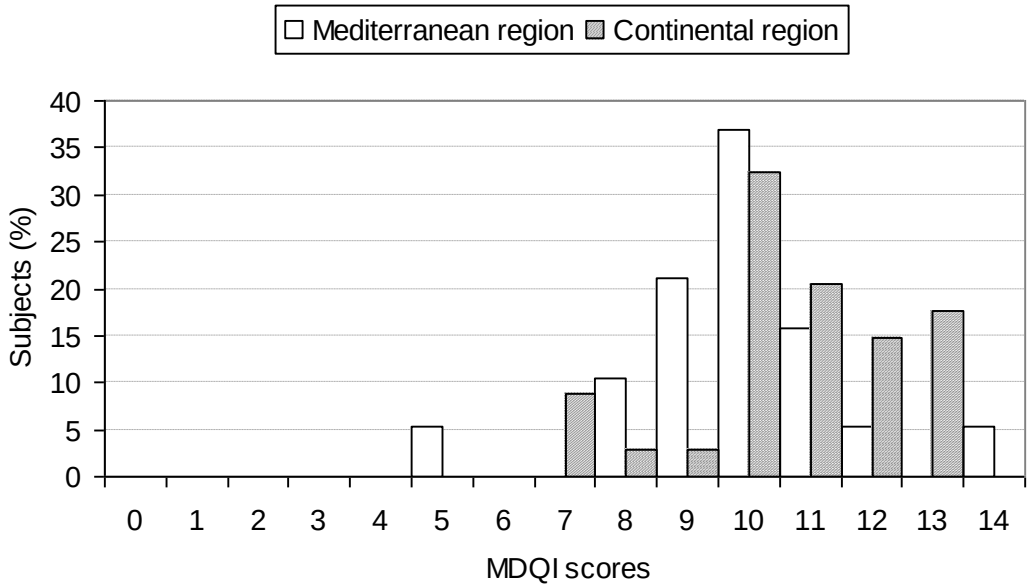

Figure 1. Distribution of subjects from Mediterranean and continental regions classified by the total MDQI score

Adherence to gluten-free diet is having a significant impact on the dietary pattern in celiac patients. Mediterranean diet as a synonym for healthy and balanced diet may serve as a healthy pattern for this sensitive population. To achieve healthy dietary pattern celiac patients should decrease intake of saturated fatty acid and increase the intake of olive oil, fish, fresh fruits and vegetables, as well as naturally gluten-free cereals.

CONCLUSIONS

In conclusion, celiac patients adhering gluten-free diet have lower diet quality according to MDQI. Celiac patients living in Mediterranean region did not have better overall diet quality comparing to celiac patient from continental region. The only difference in the diet pattern was observed for saturated fatty acid, whereas subjects from continental region had statistically higher intake of saturated fatty acid than subjects from Mediterranean region.

ACKNOWLEDGEMENTS

This research was financially supported by the Ministry of Science, Education and Sports of the Republic of Croatia through project 058-0222411-2820 'Diet, homocysteine and bone health'.

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THYMOL DEPLETION IN THE PIG STOMACH

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ABSTRACT: Aromatic plants and their extracts have been used in human and veterinary medicine since ancient times. However, the exact mode of action and kinetics or these compounds are generally poorly understood. The aim of this work was to determine the degradation and absorption kinetics of thymol in the pig stomach. An improved GC method was developed to easily measure the thymol content in samples extracted into ethylacetate. The GC separation was achieved on a 30 m × 0.25 mm × 0.25 mm film thickness Equity™ - 1701 fused silica capillary column, resulting in a standard curve over 1–500 mg/L. Isolated pig stomach was incubated for 360 min at 37 °C in an incubator with 95% O_2:5% CO_2 atmosphere on an oscillating plate at 40 rpm. Tyrode's solution pH 6.5 was used as incubation fluid. Pig stomach contents were inoculated with thymol (1.7 mM), and samples were collected at intervals during incubation and were extracted as is or after treatment with a lysis buffer. The lysis buffer was used to release thymol that may have been internalized by endogenous bacteria. Thymol concentrations in unlysed stomach samples decreased rapidly, being reduced more than twofold during 30 min incubations. Conversely, thymol concentrations in lysed samples increased rapidly, following 60 min incubations, concentrations were achieved at twice those measured in unlysed stomach contents at the beginning of the incubation. Thymol concentrations in lysed samples remained nearly double those in unlysed samples even after 360 min. Differing thymol concentrations measured in lysed and unlysed samples suggest luminal depletion resulting from bacterial uptake of thymol. Upon ingestion, luminal depletion would be expected to occur rapidly in stomach contents, but whether this sequestration would make thymol unavailable for absorption in the stomach or more distally is not known. Further investigation is needed to identify the bacteria responsible for this "thymol sequestration" phenomenon.
Key words: *gas chromatography, thymol, pig stomach*

INTRODUCTION

In the past, antibiotics provided in small amounts were used to halt the growth of bacteria, prevent outbreaks of diseases and enhance the rate of growth in intensively reared animals. In recent years the use of antibiotics as growth promoters in the European Union (EU) countries has been curtailed by legislation (Dibner and Richards, 2005). The reason for this ban has been the concern over potential development of resistant bacterial strains, which was clearly shown in the case of the *Salmonella enterica* serovar Typhimurium DT104 outbreak in the late 1990's (Mølbak *et al.*, 1999), and the potential of transferring resistance to other strains of bacteria. There is concern that if pathogenic bacteria gain resistance to antibiotics used against human infections, then these infections may become untreatable. Therefore, finding alternatives to antibiotic growth promoters has currently become the ultimate goal. Candidates used to replace antibiotic growth promoters must be evaluated for their ability to kill bacteria, assessed for successful delivery to the animal gut, and evaluated for their ability to enhance feed efficiency. Aromatic plant extracts and plant based products offer an opportunity in this regard, as many plants produce secondary metabolites which have antimicrobial or antiparasitic properties. Certain of these active components are generally recognized as safe for human and animal consumption in the USA, which has

prompted scientists to examine their potential to improve production efficiency and health in livestock. Aromatic plants and their extracts have been used in human and veterinary medicine since ancient times. However, the exact mode of action and kinetics of these compounds are generally poorly understood (FDA, 2004).
It was demonstrated that thymol can reduce the growth, survivability and ammonia production of *Campylobacter jejuni* and *Campylobacter coli* (Anderson *et al.*, 2009a). Food-producing animals can be reservoirs for *Campylobacter*, a leading bacterial cause of human foodborne illness. *Campylobacter* differ from other gut bacteria in that they have a limited capacity to ferment carbohydrates but can utilize amino acids as a major energy source, a process that can be inhibited by thymol (Anderson *et al.*, 2009b). Previous *in vitro* fermentations of pig gut demonstrated that a concentration of thymol at 258 mg/L, carvacrol at 255 mg/L, eugenol at 223 mg/L or *trans*-cinnamaldehyde at 56 mg/L reduced the number of total anaerobic bacteria compared to the control with a probability of 99.7% (Michiels *et al.*, 2009). Michiels *et al.* (2008) found that carvacrol and thymol were very effective against coliform, lactobacilli and streptococci bacteria, while *trans*-cinnamaldehyde mainly inhibited the growth of coliform bacteria. The same authors reported that in cecal incubations carvacrol, thymol, eugenol and cinnamon oil showed a high-efficacy against *S. enterica* Typhimurium DT104, *Escherichia coli* O157:H7 and *E. coli* K88, while showing little inhibition towards pig endogenous lactobacilli and bifidobacteria.
Other than the work of Michiels *et al.* (2008) who studied degradation of thymol in piglet intestines, no data has been published on absorption of thymol in the pig stomach when thymol was administered orally. Information on thymol absorption is necessary for application and dosage of thymol in pig feeds and also with regard to potential resistance/tolerance development of intestinal bacteria to thymol. The aim of this work was to determine the degradation and absorption kinetics of thymol in the pig stomach.

MATERIAL AND METHODS

Isolated pig stomach was incubated for 360 min at 37 °C in an incubator with 95% O_2:5% CO_2 atmosphere on an oscillating plate at 40 rpm. Tyrode's solution pH 6.5 was used as incubation fluid. Thymol (1.7 mM) was inoculated internally into the stomach contents and samples were collected at intervals during incubation, and were extracted as is or after treatment with a lysis buffer. The lysis buffer was used to release thymol that may have been internalized by endogenous bacteria.

Incubation procedure

Isolated pig stomach was placed into 5000-mL flasks containing 2000 mL of Tyrode's solution, and the pH was adjusted to 5.8. Thymol (100 mg/mL in absolute ethanol) was inoculated into the stomach contents to a final concentration of 1.7 mM. The flask was incubated for 360 min at 37 °C in an incubator with a modified atmosphere (95% oxygen, 5% CO_2), while simultaneously being rotated on an oscillating plate at 40 rpm.

Sample preparation for GC analyses

Samples were collected from incubated fluid or from stomach contents at 0, 15, 30, 60, 120 and 360 min incubation time. Samples were extracted for the determination of thymol content as is or after treatment with a lysis buffer. Lysis buffer was used to release thymol that may have been internalized by endogenous bacteria. The extraction method used to extract thymol from samples was a modification of the method used by Michiels *et al.* (2008). Briefly, extraction of thymol as is was carried out in 2.0 mL microcentrifuge tubes. Absolute ethanol (15 µL) and ethyl acetate (1 mL) were added to 0.25 mL of each sample. The mixture was vortexed for 30 sec and then left to equilibrate overnight at 8 °C. The extraction mixtures were then centrifuged at 10,000 rpm for 5 min at 25 °C. The supernatant was removed for GC analyses. Samples for the determination of the thymol internalization by endogenous bacteria were first treated with lysis buffer (1:1) and then later extracted as described.

Thymol determination by gas chromatography (GC)

The GC methods of Nozal *et al.* (2002) and Michiels *et al.* (2008) were modified and carried out on a HP 6890 GC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a capillary split/splitless inlet, total electronic pneumatic control of gas flow, autosampler and flame ionization detector (FID). A 30 m × 0.25 mm × 0.25 μm film thickness Equity™-1701 fused silica capillary column (#28372-U, Supelco, Bellefonte, PA, USA) was utilized for the GC separation of thymol. Hydrogen was used as the carrier gas at 1 mL/min. The mode used was split, with a split ratio of 5:1. Initial oven temperature was set at 130 °C and held for 1 min. A double temperature ramp program was used at the rate of 6 °C/min to 180 °C, then increased at 40 °C/min to 230 °C with a hold time of 4 min. Finally, the oven was brought back to 130 °C with a hold of 1 min, resulting in a total run time of 14.58 min. The injector and FID detector temperatures were 250 and 280 °C, respectively. Helium was used as makeup gas for the FID. Chromatographic data were collected and analyzed using the Agilent ChemStation Software (Rev. B.03.02 [341]). Results are expressed as % of thymol recovery.

RESULTS AND DISCUSSION

The results of thymol recovery in pig stomach content during incubation at 37 °C, for a period of 360 min, are shown in Figure 1. As it can be seen in Figure 1, thymol concentrations decreased in both lysed and unlysed stomach samples during incubation.

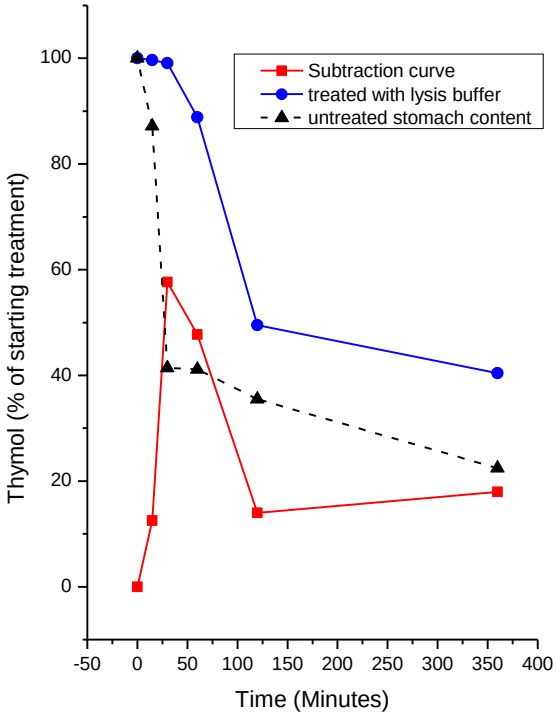

Figure 1. The concentration of thymol in the stomach contents during incubation at 37 °C. The starting thymol concentration of untreated and lysed stomach contents was 252.9 and 253.8 mg/L, respectively.

However, thymol concentrations declined more rapidly in the unlysed than in the lysed samples, with 99.1% of the added thymol recovered from lysed samples collected after 30 min incubation and only 41.4% thymol recovered from unlysed samples collected after 120 min incubation. Thymol concentrations in lysed samples (40.4% recovery) remained nearly double those in unlysed samples (22.4% recovery) even after 360 min. When presented as the difference (■) between thymol concentrations measured in lysed samples (reflecting both intracellular and luminal thymol recovery) and unlysed samples (reflecting only luminal thymol recovery), we can see that considerable amounts of thymol are initially taken up and sequestered by bacterial cells (Figure 1). This sequestration results in 57.7% of the starting thymol concentration at 30 min from the start of incubation. This intracellular sequestration appears to be transient and likely reflects leakage of thymol following thymol-caused cell wall disruption, a proposed mechanism of action of thymol and other essential oils (Burt, 2004).
Our results are different from the results of Broudiscou *et al*. (2007) who found no degradation of thymol over 24 h during *in vitro* caprine ruminal fermentation at a dose of 2000 mg/L. However, it needs to be mentioned that Broudiscou *et al*. (2007) investigated caprine rumen thymol degradation, and we have investigated pig stomach thymol degradation so these two results are difficult to compare. Varel (2002) found that thymol and carvacrol were stable in swine waste under anaerobic conditions (nitrogen atmosphere) for 62 days, with 90–95% of the thymol being recovered. Although this finding is different than our findings, it does indicate that different bacteria are present in the pig stomach and swine waste, which emphasizes the need to further identify the microorganisms responsible for thymol luminal depletion. Michiels *et al.* (2008) found that thymol was not significantly degraded in the pig jejunal gastrointestinal tract *in vitro* fermentation simulations, but significant losses of up to 29% were found in cecal *in vitro* fermentation simulations. The results of Michiels *et al.* (2008) can be justified by the fact that we have measured significant "thymol sequestration" in the pig stomach; therefore, the "sensitive" microorganisms which may uptake thymol are expected to be dead in the proximal part of the intestines. The findings of Anderson *et al.* (2009b) are similar, who evaluated palatability issues pertaining to feeding thymol. Growing pigs were provided *ad libitum* access to standard growing diets supplemented with or without 0.0067% or 0.0201% thymol on a dry weight basis. No effect of the treatment was observed on ileal or cecal *Campylobacter* or on cecal total cultureable anaerobes as well as on the accumulation of major fermentation end products within the collected gut contents. These findings suggested that appreciable quantities of thymol were absorbed or degraded in the proximal alimentary tract and that new encapsulation technologies will be needed to deliver effective concentrations of these compounds to the lower gut to achieve *in vivo* efficacy against *Campylobacter*.
No thymol was determined in the incubation fluid and suggested that no thymol uptake by the stomach itself or passive transfer to incubation fluid occurred in this *in vitro* trial. Therefore, we hypothesize that significantly different thymol concentrations measured in lysed and unlysed samples of the stomach contents are the result solely from bacterial uptake of thymol and not from absorption in the stomach. This hypothesis needs to be further evaluated and confirmed by *in vivo* studies.

CONCLUSIONS

The overall results of our trial confirmed that thymol was not absorbed in the pig stomach. However, different thymol concentrations measured in lysed and unlysed samples suggest that upon ingestion, significant amounts of thymol were internalized by endogenous bacteria. This luminal depletion would be expected to occur rapidly in stomach contents but it is not known whether this sequestration would make thymol unavailable for absorption in the

stomach or more distally. Further investigations are needed to identify the bacteria responsible for this “thymol sequestration” phenomenon.

ACKNOWLEDGEMENTS

This research was partially supported by the Norman Borlaug Institute, USA, by the USDA, ARS, SPARC, College Station, TX, USA, and by the Serbian Ministry of Education and Science, Belgrade, Serbia. Mention of trade names, proprietary products, or specific equipment is solely for the purpose of providing specific information and does not constitute a guarantee, warranty or endorsement by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

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ANTIOXIDANT COMPONENTS AND PROPERTIES OF BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH) GRAIN FRACTIONS

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ABSTRACT: Buckwheat (*Fagopyrum esculentum* Moench) is an alternative crop belonging to the *Polygonaceae* family. In comparison to antioxidant activity of frequently used cereals, buckwheat has been reported to possess higher antioxidant activity, mainly due to high rutin content.
The objective of this work was to determine the main antioxidant compounds and antioxidant activity of buckwheat grain and fractions (wholegrain, hull and groat). Buckwheat grain fractions were extracted with ethanol/water (80/20, v/v), followed by determination of total phenolic content and DPPH radical scavenging activity. Determination of phenolic compounds and tocopherols was performed by HPLC.
Significantly higher content of total phenolics was found in buckwheat hull than in wholegrain and groat. Protocatechuic acid, siringic acid, ferulic acid, sinapic acid, rutin and quercetin were quantified in all tested fractions, whereas vanilic acid was found in wholegrain and hull. The content of total tocopherols in investigated samples ranged from 9.95 μg/g for hull to 25.8 μg/g for groat. Hull was superior in scavenging activity on DPPH radicals as evidenced by its lower IC_{50} value.
Obtained results can broaden the utilization of buckwheat, especially a share of hull in wholegrain flour production. Bakery products containing wholegrain buckwheat flour may be regarded as health-promoting functional foods.
Key words: *buckwheat grain fractions, phenolics, tocopherols, DPPH radicals*

INTRODUCTION

Buckwheat (*Fagopyrum esculentum* Moench) is highly nutritious pseudocereal known as a dietary source of protein with favorable amino acid composition and vitamins (Bonafaccia et al., 2003a), starch and dietary fiber (Skrabanja et al., 2004), essential minerals (Steadman et al., 2001) and trace elements (Bonafaccia et al., 2003b). Phenolic compounds are also found in abundance in buckwheat, including rutin, orientin, vitexin, quercetin, isovitexin, kaempferol-3-rutinoside, isoorientin, and catechins (Dietrych-Szostak and Oleszek, 1999). A number of studies have shown that buckwheat possesses strong antioxidant activity, mainly due to high rutin content (Kreft et al., 2006). Although other parts of the buckwheat plant can be used for human consumption, buckwheat is now mainly grown for the production of seeds (Wijngaard and Arendt, 2006). Buckwheat seeds are usually processed into flour, and they are dehulled before milling or the flour is sieved afterward.
Hung and Morita (2008) investigated 16 buckwheat milling fractions and concluded that the phenolic acids and flavonoids are mainly located in the outer layers of grain.
In our previous studies (Sedej et al., 2010; Sedej at al., 2011), significantly higher content of phenolic compounds and tocopherols was found in wholegrain buckwheat flour than in refined one. Wholegrain buckwheat flour possessed better scavenging abilities on 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$) radicals, as well as better reducing activity, antioxidant activity (AOA) by β-carotene bleaching method, and chelating activity on Fe^{2+}, according to their IC_{50} values.
The objective of this work was to investigate antioxidant capacity of buckwheat hull in comprasion to wholegrain and groat in order to get insight into the possibility of hull

supplementation of wholegrain buckwheat flour, resulting in improved ingredient of functional foods.

MATERIAL AND METHODS

Materials

Buckwheat (*Fagopyrum esculentum* Moench) grain fractions: wholegrain (10.0% water content, 1.93% db ash content, 12.4% db protein content), hull (8.33% water content, 1.44% db ash content, 4.11% db protein content) and groat (9.97% water content, 2.11% db ash content, 14.2% db protein content) were obtained from Hemija Commerce, Novi Sad, Serbia. Buckwheat grains were imported from Ukraine and dehulling was carried out at Jazak Mill, Serbia.

Extraction Procedure

Buckwheat grain fraction (50.0 g) was mixed with 200 mL of ethanol/water (80/20, v/v). Extraction was carried out by shaking the mixture at room temperature for 1 h, supernatant was filtered through the filter paper (Whatman, Grade 4 Chr, UK), and the procedure was repeated twice. Combined extracts were dried by vacuum-evaporator. The dried extract was resolved in ethanol/water (80/20, v/v) to 10 mL volume. The extract obtained by this procedure was used for further investigation of total phenolic content, and antioxidant activity.

Determination of Total Phenolic Content

Total phenolic content of buckwheat grain fractions extracts was determined spectrophotometrically at 750 nm by using Folin-Ciocalteu's reagent (Singleton et al., 1999). Gallic acid was used as a standard and results were expressed as gallic acid equivalents (GAE) (μg GAE per g of sample on wet mass basis).

HPLC Determination of Phenolic Compounds

Five grams of buckwheat grain fraction was extracted with 20 mL methanol/water (90/10, v/v) at room temperature during 24h, ultrasonicated for 10 min and filtered through 0.45 μm pore size PTFE filter (Rotilabo-Spritzenfilter 13 mm, Roth, Karlsruhe, Germany) before injection into the HPLC system.

HPLC analysis was performed by using a liquid chromatograph (Agilent 1200 series), equipped with a diode array detector (DAD), on an Agilent, Eclipse XDB-C18, 1.8 μm, 4.6 x 50 mm column, at a flow-rate of 1.000 mL/min. A single rapid resolution reverse phase HPLC method suitable for the determination of 17 phenolic compounds was developed and validated as previously reported (Mišan et al., 2011).

HPLC Determination of Tocopherols

Extraction of tocopherols from the samples and HPLC analysis were carried out according to the method of Sedej et al. (2010).

DPPH Radical Scavenging Activity

Effect of the examined extracts on the content of DPPH radicals was estimated according to the method of Hatano et al. (1988). Buckwheat grain fractions extracts at various concentrations (1.00–5.00 mg/mL) were tested and the absorbance of the reaction mixture was measured at 517 nm. The IC_{50} value (mg/mL) was defined as the concentration of an antioxidant extract which was required to quench 50% of the initial amount of $DPPH^{\bullet}$ under the experimental conditions given. Butylated hydroxytoluole (BHT) was used as a control.

Statistical Analysis

All analyses were performed in triplicate, and the mean values with the standard deviations are reported. Analysis of variance and Duncan's multiple range test were used. Statistical data analysis software system STATISTICA (StatSoft, Inc. (2011) data analysis software

system, version 10.0. www.statsoft.com) was used for analysis. *P* values < 0.05 were regarded as significant.

RESULTS AND DISCUSSION

Total Phenolic Content

Total phenolic content of buckwheat grain fractions extracts is shown in Figure 1. The total phenolic content of buckwheat grain fractions differed significantly ($P < 0.05$) ranging from 1.69 ± 0.42 mg GAE/g in groat to 2.09 ± 0.42 mg GAE/g in hull (Figure 1). Results with similar trend of increased content of phenolics in outer layers of grain were obtained in the studies of Hung and Morita (2008). The results of Velioglu et al. (1998) confirmed higher level of phenolic content in buckwheat hull than in seeds.

Figure 1. Total phenolic content of buckwheat grain fractions extracts (mg GAE/g sample)
Values are means of three determinations ± standard deviation.
Bar values with the same superscript are not significantly different (P < 0.05).

HPLC Determination of Phenolic Compounds

Using HPLC-DAD, rutin was determined as major compound in crude methanolic extracts of buckwheat grain fractions. Protocatechuic, syringic, ferulic, and sinapic acid, and quercetin were quantified in all tested fractions, whereas vanilic acid was found in wholegrain and hull (Table 1).

Table 1. Phenolic compounds of buckwheat grain fractions (µg/g)

Phenolic compounds	Wholegrain	Hull	Groat
Protocatechuic acid	92.6 ± 0.94^{a}	168 ± 0.48^{c}	103 ±1.21^{b}
Vanilic acid	15.0 ± 0.46^{a}	37.1 ± 2.38^{b}	n.d.
Syringic acid	52.3 ± 1.49^{b}	36.3 ± 1.6^{a}	63.5 ± 0.35^{c}
Ferulic acid	17.2 ± 0.13^{a}	17.5 ± 0.59^{a}	17.4 ± 0.21^{a}
Sinapic acid	28.13 ± 1.68^{a}	29.8 ± 0.47^{a}	30.4 ± 3.44^{a}
Rutin	146 ± 4.56^{b}	225 ± 7.25^{c}	116 ± 0.26^{a}
Quercetin	3.04 ± 0.14^{a}	33.2 ± 1.74^{b}	2.48 ± 0.06^{a}
Total	354.5	546.9	332.8

Values are means of three determinations ± standard deviation. Values in each row with the same superscript are not significantly different (P < 0.05). n.d.-not detected

Hung and Morita (2008) published that rutin and ferulic acid were the most represented phenolic compounds in 16 buckwheat milling fractions. In addition, Alvarez-Jubete et al. (2010) identified syringic acid and caffeic acid in buckwheat grain. In buckwheat groat vanilic acid, syringic acid, ferulic acid, and coummaric acid were quantified (Zielinski et al., 2006).

The highest content of all identified compounds, with an exception of syringic acid, was established for hull. Total phenolic compounds content of buckwheat grain fraction was present in the following order: hull > wholegrain > groat. This trend is in accordance with results of Gallardo et al. (2006), who concluded that milling and fractioning of grains greatly influenced the phenolic composition of the final milled product. The highest concentration of rutin in hull, and similar trend of rutin and quercetin distribution in buckwheat milling fractions was confirmed by Steadman et al. (2001).
Content of phenolic compounds determined by HPLC is in significant correlation with the results obtained for Folin-Ciocalteu method (r = 0.87, P < 0.05).

Tocopherols Content

Grains are relatively good sources of vitamin E, α-, β-, γ-, δ-tocopherols and tocotrienols, and therefore content of α-, γ-, δ-tocopherols is determined in buckwheat grain fractions. The results are shown in Table 2. The content of total tocopherols in buckwheat grain fractions ranged from 9.95 μg/g for hull to 25.8 μg/g for groat.
The highest content of α-tocopherol was found in wholegrain. Content of γ-tocopherol was significantly higher in both wholgrain and groat in comparison to hull. Within the grain, tocopherols are primarily located in the germ and aleurone tissue, the metabolically active parts of grain (Engelsten and Hansen, 2009). The data show that the content of tocopherols strongly depends on the milling process, namely refining process of flour, in which the outer layer of grain is removed.

Table 2. Tocopherol content of buckwheat grain fractions (μg/g)

Buckwheat	α-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total
Wholegrain	9.44 ± 0.92^{c}	10.1 ± 0.89^{b}	0.37 ± 0.05^{a}	19.8
Hull	8.13 ± 1.07^{b}	1.36 ± 0.11^{a}	0.46 ± 0.01^{a}	9.95
Groat	3.32 ± 0.04^{a}	22.1 ± 0.2^{c}	0.42 ± 0.03^{a}	25.8

Values are means of three determinations ± standard deviation. Values in each column with the same superscript are not significantly different (P < 0.05)

Antioxidant Activity

All extracts showed significant (P < 0.05) difference in their ability to reduce the initial concentration of DPPH$^{\bullet}$ which was confirmed by their IC_{50} values (Figure 2). The hull extract showed higher DPPH$^{\bullet}$ scavenging activity in comparison with reference antioxidant, BHT as evidenced by its lower IC_{50} value. Scavenging activity on this radical was 18-fold higher for buckwheat hull (0.39 ± 0.02 mg/mL) than for groat (1.66 ± 0.01 mg/mL). These findings are in accordance with published results of Hung and Morita (2008) who reported that the outer layers of grains contain higher total phenolics contents and possess significantly higher antioxidant capacity than the inner fractions when the DPPH assay was used. Also, Sun and Ho (2005) reported powerful antiradical activity of ethanolic extract of the whole buckwheat grain on DPPH$^{\bullet}$, but our results are not fully comparable because of differences in applied methods.

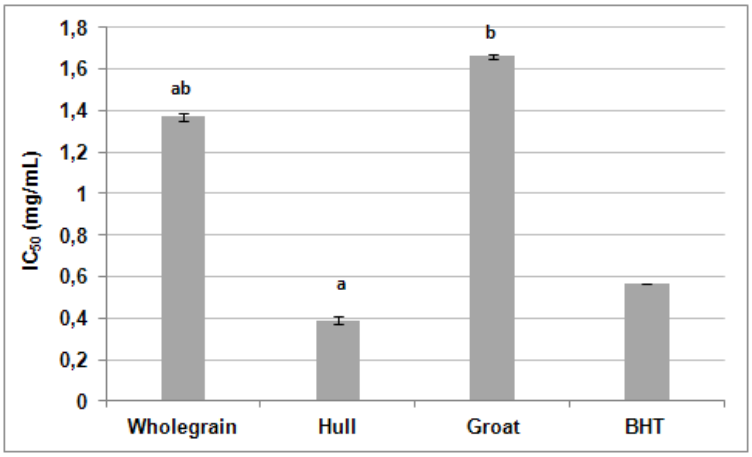

Figure 2. Scavenging activity of buckwheat grain fractions extracts on DPPH$^{\bullet}$ radicals, expressed as IC_{50} (mg/mL)
Values are means of three determinations ± standard deviation.
Bar values with the same superscript are not significantly different (P < 0.05)

CONCLUSIONS

The results of this paper confirm that buckwheat hull is superior in antioxidant compounds content and antioxidant activity in comparison to wholegrain and groat. These findings suggest the potential improvement of the antioxidant properties of wholegrain buckwheat flour, and consequently bakery food products through addition of hull. The suggested improvement has significant implication for use of by-product and design of cereal-based functional foods.

ACKNOWLEDGEMENTS
This work is a part of the Project (TR–31029) supported by the Ministry of Education and Science, Republic of Serbia.

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URINARY LIPID OXIDATION BIOMARKERS ARE INFLUENCED BY FATTY ACID INTAKE AND NOT BY OTHER DIETARY FACTORS

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ABSTRACT: Oxidative stress is characterized as imbalance between the content of free radicals and antioxidants in favour of radicals. Dietary nutrients may limit or encourage lipid damage therefore influence urinary oxidative stress biomarker excretion. The aim of this study was to evaluate nutrient intake in comparison to reference values (DACH, 2004) and to examine the influence of daily nutrient intake, as positive and negative factors, on urinary levels of malondialdehyde (MDA) and 4-hydroxy2nonenal (4HNE) in healthy human. Only one subject participated in this pilot nutritional study, which was undertaken using the food diary weighing method for 15 consecutive days. Nutrient intake was assessed with Prodi 5.7 Expert plus (Kluthe, 2010) computer nutritional programme. Morning urine samples were used for lipid oxidation marker analysis and normalized with creatinine values. MDA and 4HNE were analysed by GC/MS system. Results were statistically evaluated by using PASW Statistics 18 programme. The results showed that average daily macronutrient intake in terms of energy caloric profile was sufficient, but not in compliance with DACH Reference for Nutrient Intake. MDA and 4HNE levels were on average 25.85±9.51 and 0.31±0.30 ng/mg creatinine, respectively. Normalized MDA and 4HNE were correlated (r=0.52), but not statistically significant. Positive factors such as antioxidant vitamins, total dietary fibre and β-carotene showed no influence on MDA neither on 4HNE values. However there was a statistically significant correlation between PUFAs and 4HNE (r=0.620) compared to MDA, where correlation was not found. Moreover there was also significant correlation between linoleic acid, as major precursor for lipid oxidation, and 4HNE urinary excretion (r=0.642). Intake of PUFAs and linoleic acid as a substrate for lipid oxidation had influence on urinary excretion of 4HNE, but not on MDA. None of the expected positive factors showed statistically significant effect on analyzed biomarkers.
Key words: *nutrient intake, healthy human, oxidative stress, urinary lipid peroxidation biomarkers*

INTRODUCTION

In observational studies, high intake of fruit and vegetables is frequently associated with decreased risk of coronary heart disease and many cancers (Riboli and Norat, 2003; Dauchet et al., 2006). Various plant-derived compounds may have direct protective effects in tissues or enhance cellular defence mechanisms and thus restrain pathophysiological processes associated with degenerative diseases (Liu, 2003). The inverse association between the intake of fruit and vegetables and the risk of degenerative diseases has often been at least partly attributed to the high content of antioxidants in plants. The role of diet in pro- and antioxidative processes is an intriguing concept and, at least in theory, diet can both decrease (for example, by antioxidant vitamins; McCallnd Frei, 1999; Frei, 2004) and increase (for example, by polyunsaturated fatty acids, PUFA; Turpeinen et al., 1998; Eritsland, 2000) oxidative stress in the body. The assessment of oxidative stress status though specific biomarkers has acquired great importance. Measurement of antioxidant capacity may also involve the assessment of specific oxidative stress biomarkers. Most of the studies that have examined the association between diet and oxidative stress consider the effects of antioxidant supplements (vitamins and minerals), drinks and foods with bioactive compounds or dietary patterns on oxidative stress biomarkers. The major biomarkers include the products of the attack of free radicals and reactive species to various substrates: lipids, proteins and nucleic acids (Barbosa et al., 2008).

Lipid peroxidation proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids (PUFA), because they contain multiple methylene interrupted double bonds (Guichardant, 2009). Several markers of lipid peroxidation are available with different degrees of specificity, from malondialdehyde (MDA) as a global marker, to F2-isoprostane, which is specifically produced from arachidonic acid. Among these, 4-hydroxynonenal (4-HNE) is recognized as a breakdown product of fatty acid hydroperoxides, such as 15-hydroperoxy-eicosatetraenoic acid and 13-hydroperoxy-octade cadienoic acid from the *n*-6 fatty acids (Guichardant, 2004). These biomarkers, measurable in urine, are noninvasive that give an indication of the oxidative stress. Taking into account the limitations of the different biomarkers available, more than one biomarker should be measured in a particular sample to obtain an overview on the oxidative stress (Guichardant, 2009).
The aim of this study was to evaluate nutrient intake in comparison to reference values (DACH) and to examine the influence of daily nutrient intake, as positive and negative factors, on urinary levels of malondialdehyde (MDA) and 4-hydroxy2nonenal (4HNE) in healthy human.

MATERIAL AND METHODS

This pilot nutritional study was done on only one healthy 29 years old female subject (weight 55 kg, high 169 cm). She completed food intake diary using the weighing method for 15 consecutive days. Nutrient intake was assessed with Prodi 5.7 Expert plus (Kluthe, 2010) computer nutritional programme. Participant collected each morning urine samples while recording food intake. Aliquote urine samples were stored in -80°C until analysis. 4-hydroxy2nonenal (4HNE) and malondialdehyde (MDA) analysed by GC/MS system were used as urinary lipid oxidation biomarkers. The measured 4HNE and MDA concentrations were normalized using the corresponding creatinine levels in each sample. Results were statistically evaluated by using PASW Statistics 18 programme.

4HNE and MDA determination

4HNE and MDA aldehydes were determined together with slightly modified stir bar sorptive extraction-thermal desorption-gas chromatography/mass spectrometry method (Stopforth et al., 2006). 4-hydroxy nonenal-d_3 (Cayman Chemical) was used as internal standard. Stir bar sorptive extraction (SBSE) was done using twister stir bar (10 mm x 1 mm x 1 mm d_f polydimethylsiloxane (PDMS)) from Gerstel. PFBHA in ultrapure water was used for derivatization procedure. GC/MS was carried out on an Agilent 7890 gas chromatograph that was interfaced with an Agilent 5975C mass selective detector. The derivatives were separated on a DB-5 capillary column (30 m x 0.25 mm x 0.25 µm, Agilent) using helium as carrier gas at a flow rate of 1mL min^{-1}.

RESULTS AND DISCUSSION

Average daily intake of energy is 7254±1260 KJ, which is under the lower limit for healthy female between 25 to 51 years old (PAL = 1.4), but still in accordance with DACH reference values. Total daily protein intake is with 1.12 g/kg BW statistically significantly different than recommendations (DACH, 2004). The amount of the dietary energy supplied by carbohydrates is on average 47.2% which is significantly lower than reference values (2004), consequently fats (35 % of energy) are significantly higher. Average daily intake of dietary fiber is with 27.1±9.4 g/day in compliance with recommendations (DACH, 2004). Total amount of vitamins A and E consumed on average is the same as DACH recommendations, whereas vitamin C is statistically significantly higher (Table 1).
On average urinary MDA and 4HNE levels were 25.85±9.51 and 0.31±0.30 ng/mg creatinine, respectively (Figure 1). MDA level in morning urine samples varied between 13.1 (day 12) and 46.4 (day 6) ng/mg creatinine. Morning 4HNE also peaked at day 6 with 0.96 ng/mg creatinine, while it was not detected on day 10 and 14, minimum was on day 15 (0.03 ng/mg

creatinine). Interestingly, 4HNE concentrations at the beginning of the trial (first six days) were significantly higher than the following nine days, whereas MDA was dispersed during the whole trial. Normalized MDA and 4HNE were correlated (r=0.52), but not statistically significant.

Morning spot urine levels of MDA and 4HNE were compared to all macronutrients and antioxidant vitamins, as well as fatty acids as aldehyde oxidation precursors. 4HNE values on day 10 and 14 were excluded from correlation calculations due to levels below the detection limit. Dietary nutrient intake values were compared to the next day morning urine sample biomarkers.

Table 1. Average nutrient intake in comparison to Reference Values for Nutrient Intake (2004)

	Mean	Std. Deviation	DACH Reference Value for Nutrient Intake (2004)	Sig. (α = 0.05)
Nutrient				
Energy (KJ)	7254	1260	7800	0.115
Charbohydrates (% energy)	47.24	6.86	55	0.001
Proteins (g/kg BW)	1.12	0.28	0.8	0.001
Fat (% energy)	34.98	7.58	30	0.023
PUFA (% energy)	6.66	2.87	7	0.649
SFA (% energy)	11.68	3.49	10	0.083
TDF (g)	27.11	9.37	30	0.252
Vit A (µg retinol equivalent)	859.9	288.2	800	0.434
Vit E (mg tocopherol equivalent)	11.13	3.53	12	0.356
Vit C (mg)	152.39	61.47	100	0.005

BW – body weight; PUFA – polyunsaturated fatty acids; SFA – saturated fatty acids; TDF – total dietary fibre

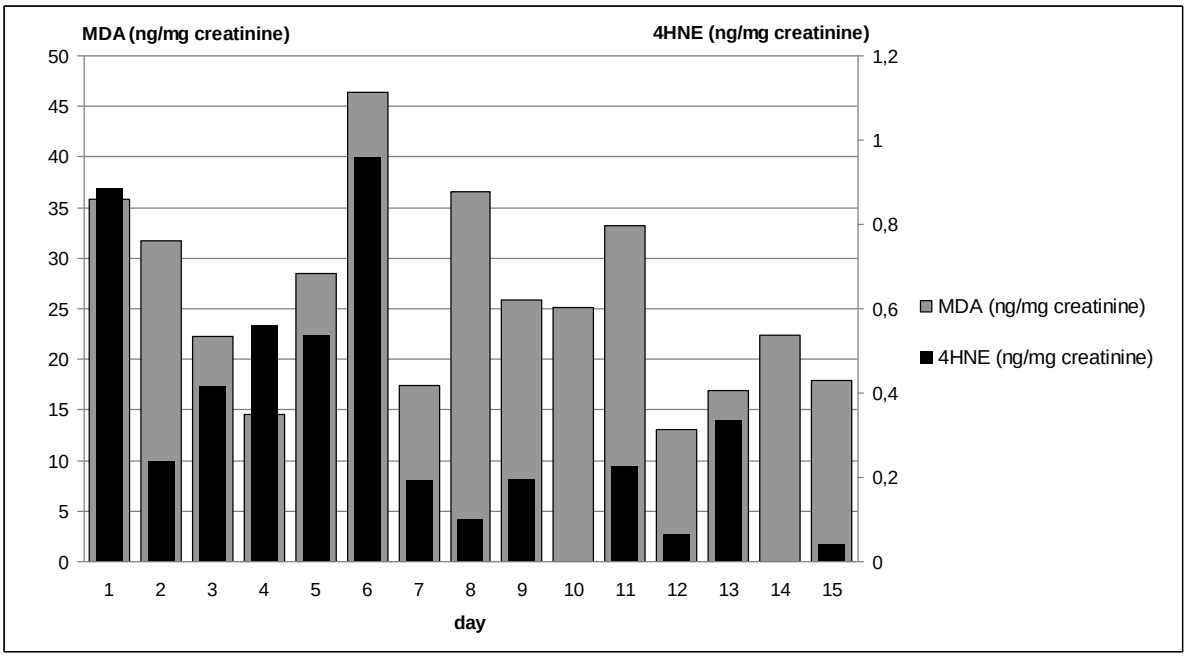

Figure 1. MDA and 4HNE concentration in morning urine samples during the trial

Positive factors such as antioxidant vitamins, total dietary fibre and β-carotene had no influence on MDA neither on 4HNE values. Results showed statistically significant correlation between PUFAs and 4HNE (r=0.620) in comparison to MDA where correlation was not found. Moreover there was also significant correlation between linoleic acid, as major precursor for lipid oxidation, and 4HNE urinary excretion (r=0.642) (Figure 2). Pearson coefficient correlations showed that levels of excreted 4HNE was more influenced by fatty acid intake than by any positive dietary factors, whereas with MDA this was not the case. Therefore, when researching lipid oxidation biomarkers, fatty acid intake must be taken into account prior to dietary intake of antioxidants.

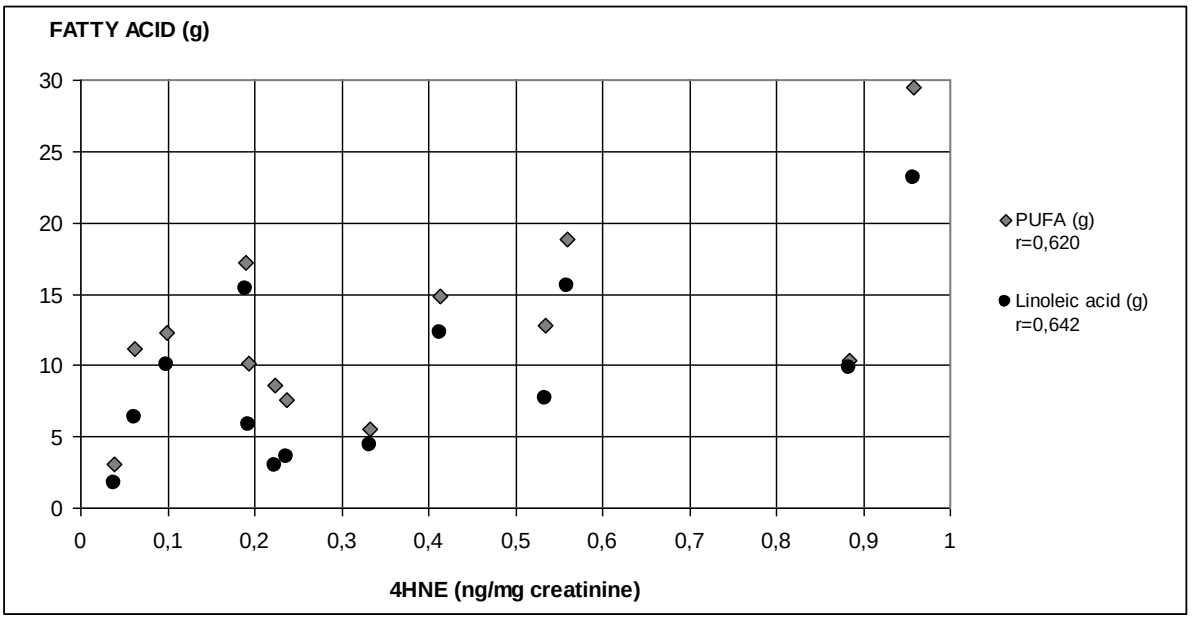

Figure 2. Morning urinary 4HNE excretion in correlation with daily PUFA and linoleic acid intake

CONCLUSIONS

Average daily macronutrient intake in terms of energy caloric profile was sufficient, but not in compliance with DACH Reference for Nutrient Intake. Intake of PUFAs and linoleic acid as a substrate for lipid oxidation had influence on urinary excretion of 4HNE, but not on MDA. None of the expected positive factors showed statistically significant effect on analyzed biomarkers. No apparent agreement could be made between the intake of antioxidants and the reported urinary 4HNE and MDA concentrations.

ACKNOWLEDGEMENTS

This study was partially financially supported by the European Union within the European Social Fund.

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DIET AND HEAT TREATMENT EFFECT ON FATTY ACID COMPOSITION IN DIFFERENT PIG TISSUES

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ABSTRACT: Linseed is one of the most useful crops that has been cultivated as a commercial plant all over the world. Recently there has been a growing interest in linseed oil due to the high concentration of linoleic and especially α-linolenic acid. Since animals are not able to synthesize these essential fatty acids, changes in fatty acid content in meat can be achieved through the changes in animal diet. The aim of this study was to evaluate the influence of diet supplemented with linseed rich additive on fatty acid profile and ω-fatty acids content in pig meat. Furthermore, fatty acid composition of roasted meat of pigs fed with control and experimental diet was investigated.
Twelve pigs were divided in a control and experimental group and grown to 110 kg of live weight. The experimental group was fed a standard diet enriched with 2.5% of commercial additive. Fatty acid composition of fresh meat samples and heat treated meat were determined by GC-FID. Meat was roasted in oven at the temperature of 80 - 85^{0}C until the temperature in the centre reached 69^{0}C (about 1 hour). STATISTICA software, version 10 was used for performing ANOVA and Fishers comparison of means.
Samples from the experimental group fed with linseed enriched diet showed significantly higher ($p < 0.01$ and $p = 0.01$, respectively) content of both ω-3 (8.25% vs. 1.33%) and ω-6 fatty acids (25.68% vs. 20.68%) in all tissues, thus making it better for a human consumption from a health perspective. Heat treatment significantly decreased ($p < 0.05$) the content of stearic and linoleic acid in control tissue samples, but decrease was insignificant ($p > 0.05$) in samples from the experimental group.
In conclusion, diet enriched with linseed had a beneficial effect on the majority of monitored parameters in the study.
Key words: *linseed diet, essential fatty acids, pork, heat treatment*

INTRODUCTION

Dietary fats, especially essential fatty acids, which cannot be endogenously synthesized by humans and other mammals, play an important role in human health and well being (Beare-Rogers *et al.*, 2001). Many clinical, epidemiological and biological studies ascribe a particular significance to ω-3 polyunsaturated fatty acids (PUFA). Results from those studies confirmed their anti-inflammatory, antithrombogenic, and hypotriglyceridemic properties; they are also active against some types of cancer like colon, breast and prostate cancer (Rose *et al.*, 1999; Connor, 2000; Simopoulos, 2002). However, these beneficial effects are limited by the fact that the modern western diet is rather low in ω-3 fatty acids, while it is very high in ω-6 fatty acids content, and it is well known fact that increased levels of ω-6 fatty acids are associated with an increase in chronic diseases (Givens *et al.*, 2006; Enser *et al.*, 2000).
Since animal diet determines the fatty acid composition in meat, changes in fatty acids ratio and composition in meat can be achieved through the changes in animal diet (Mourot, 2001). Most common way to perform this is by enriching the animal feed with fish oil/fish meals or with plant oils rich in ω-3 PUFA or seed meals such as linseed (Raes *et al.*, 2004; Kouba *et al.*, 2003). Linseed, containing about 40% of oil, has long been used in human and animal diet. Oil from the seeds contains remarkably high content of linoleic (LA, 18:2, n-6) and especially α-linolenic acid (ALA, 18:3, n-3). Furthermore, linseed is the richest oilseed source of ALA (Juárez *et al*., 2010, Łukaszewicz *et al*., 2004, Ivanov *et al.*, 2012). Monogastric animals (like pigs) are better target for this approach, because the dietary fatty acids are absorbed from the intestine unchanged (Enser *et al.*, 2000). In this way, composition of fatty

acids in meat could be modified by dietary means ultimately improving nutritional and health value of the meat.
The objective of presented study was to investigate the influence of linseed enriched diet (rich in ω-3 PUFA) on fatty acid composition of pig meat. This was done by supplementing the standard, commercial feed with linseed and observing the effects of such feeding (with standard diet used as a control) on changes in fatty acid composition of various pig tissues. Furthermore, the samples were roasted in order to obtain information about fatty acid degradation caused by the heat treatment.

MATERIAL AND METHODS

Animals and diet

The total twelve pigs, Pietrain x (Landrace x Great Yorkshire) were used in the study, which was conducted at the pig farm „Sabo Janos", Jermenovci, Serbia.
Twelve pigs were divided into two groups and fed with two types of diet, a standard diet and diet enriched with 2.5% of Vitalan® (Vitalac, France), until reaching approximate 110 kg of live weight, when they were slaughtered. Vitalan® contains 85% extruded linseed, which made the diet rich in omega-3 acids and the rest were wheat bran and antioxidants. The composition of the diets is shown in Table 1.

Table 1. Composition of diets for pigs

Components (%)	Control group	Experimental group
Vitalan®	0	2.5
Maize	51.0	50.0
Barley	28.0	26.8
Soybean meal	18.0	17.7
Premix	2.5	2.5
Acidifier	0.5	0.5
Total	100.0	100.0

Slaughtering and sampling

The animals were slaughtered and samples of pig meat (*M. Longissimus dorsi,* bacon and back fat), 3 pieces (200 g each) from both groups were collected and kept in the refrigerator at 4^0C. A half amount of every sample was roasted in the oven at the temperature of 80 - 85^0C until the temperature in the centre of the meat reached 69^0C (about 1 hour). After 24 hours, the samples were sent to the laboratories of Institute of Food Technology in Novi Sad, where fatty acid analysis was performed. Each sample was coded with a letter: C for samples from control group and E for samples from experimental group.

Fat extraction for fatty acid analysis

Supercritical fluid extraction with CO_2 was used for preparation of fat extracts, as recommended for fatty acid analysis (Ivanov *et al.*, 2012). Extractions were performed on a LECO TFA2000 fat analyzer with the method adopted from existing LECO procedures (Leco corporation, 2003). Infusorial soil (flux calcined infusorial soil, up to 54% crystalline silica, cristobalite < 50%, quartz < 4%, produced by LECO Corporation, MI, USA) was used as absorbent to remove traces of water from samples. The preselected meat samples were homogenized with food processor. 1.0 g of homogenized meat was vigorously mixed with 2.2 g of absorbent and this way prepared mixture was transferred into a metal extraction thimble (12 cm length and 10 mm diameter) for the extraction. After finishing the extraction step, the instrument was depressurized, and extracts in collection vials were de-gassed for ten minutes, until achieving constant weight of extracts.

Fatty acid determination
From the extracted lipids, fatty acid methyl esters were prepared with method that uses boron trifluoride/methanol solution (Veresbaranji, 1996). Nitrogen gas was used for drying and removing solvents from fatty acid methyl esters. Obtained samples were analyzed by a GC Agilent 7890A system with FID, auto-injection mode, equipped with fused silica capillary column (DB-WAX 30 m, 0.25 mm, 0.50 μm). Helium was used as a carrier gas (purity > 99.9997 vol. %, flow rate = 1.26 ml/min). The fatty acids peaks were identified by comparison of retention times with retention times of standards from Supelco 37 component fatty acid methyl ester mix (Cat. No. 47885-U, Supelco, PA, USA) and with data from internal data library, based on previous experiments and fatty acid methyl ester determination on GC-MS. Results were expressed as mass of fatty acid or fatty acid group (g) per 100 g of fatty acids, and as a ratio between ω-6 and ω-3 fatty acids.

Soxhlet extraction – free fat determination
Soxhlet extractions were performed with a Büchi 810 Soxhlet fat extraction apparatus (Soxtec system HT, 1043 Extraction Unit, Foss Tecator AB, Höganäs, Sweden) in accordance with manufacturer procedure and AOCS Method Ba 3-38 (AOCS, 2001). Extractions were performed with petroleum ether (40 - 60º) solvent. Sample size was 3g and extraction time was 1.5h, at 80 ºC. After removal from the apparatus, extracted lipids were allowed to cool at room temperature while passing air over the samples for 1 minute, and then dried in desiccant pouch until they reached constant weight (approximately 60 minutes). Fat content was expressed as the percentage, by weight (gravimetrical method).

Statistical analysis
STATISTICA software version 9 (Statsoft, Tulsa, OK, USA) was used for analyzing variations (analysis of variance – ANOVA), and for Fishers LSD comparison of means and pairwise comparison. Differences among means with probability (p) ≤ 0.05 were accepted as representing statistically significant differences, and differences among means with 0.05 ≤ p ≤ 0.10 were accepted as representing tendencies to differences.

Results and Discussion
Effects of the diets used in the experiment on composition of fatty acid group and total fat content of pig tissues are presented in Table 2. Samples of *M. longissimus dorsi* of the control and experimental groups showed significant differences in fatty acid composition. Saturated fatty acid (SFA) content decreased significantly ($p = 0.006$), monounsaturated fatty acids (MUFA) content also significantly ($p = 0.04$) decreased, and PUFA and unsaturated fatty acids (UFA) contents significantly ($p = 0.03$ and $p = 0.02$, respectively) increased in experimental groups. Experimental diet had less influence on back fat fatty acid composition, but increase in ω-3 fatty acid content was still significant ($p < 0.05$).

Table 2. Diet effects on composition of fatty acid group and total fat content of pigs tissues

		Fatty acids (% of total lipids)					*n-6/n-3* ratio	UI*	Total fat content (%)
		SFA	MUFA	PUFA	*n-6*	*n-3*			
Meat	C	17.28 ± 0.47	59.63 ± 0.30	23.09 ± 0.31	19.61 ± 0.26	2.49 ± 0.17	7.88	1.09	5.11 ± 0.08
	E	13.30 ± 0.38	52.17 ± 0.35	36.44 ± 0.33	24.22 ± 0.06	10.89 ± 0.35	2.22	1.37	5.57 ± 0.06
	Probability	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$			
Back fat	C	17.95 ± 0.40	57.10 ± 0.26	23.95 ± 0.34	22.36 ± 0.16	0.71 ± 0.38	31.49	1.07	86.50 ± 0.01
	E	17.73 ± 0.43	56.61 ± 0.20	24.31 ± 0.23	27.20 ± 0.17	7.20 ± 0.16	3.78	1.34	82.90 ± 0.03
	Probability	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.05$			
Bacon	C	16.98 ± 0.35	61.14 ± 0.16	21.59 ± 0.14	20.07 ± 0.13	0.79 ± 0.12	25.41	1.05	28.10 ± 0.03
	E	14.69 ± 0.30	51.23 ± 0.18	35.87 ± 0.16	26.11 ± 0.14	6.67 ± 0.16	3.91	1.25	26.10 ± 0.04
	Probability	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$			

The results are presented as mean ± SD

** UI – Unsaturation index = average number of double bonds per fatty acid residue*

Linseed diet positively affected ω-6/ω-3 ratio of all samples by decreasing it below the desirable level. Being aware of ω-3 PUFA benefits and health promoting effects, the nutritionists recommend a diet rich in ω-3 fatty acids, as well as a lower ω-6/ω-3 ratio from the currently common 15-20:1 to 1-4:1 (Gebauer et al., 2006, Raes *et al.*, 2004).
It is known that UFA containing double bonds that are easily oxidized, and thus fatty acid composition can influence the palatability of meat, as well as that the effect of fatty acids on meat tenderness is due to the different melting points of individual fatty acids, especially stearic and linoleic acid (Jeong *et al.*, 2010). Changes in the content of these two acids in *M. longissimus dorsi* are shown in Table 3, as a result of the differences in composition of standard (control) and linseed enriched diet used for feeding of pigs.

Table 3. Changes in content of stearic, linoleic and omega fatty acids in pigs *M. longissimus dorsi* muscle after roasting

		Fatty acids (% ot total lipids)			
		Stearic acid	Linoleic acid	*n-6*	*n-3*
Control diet	Fresh	7.33 ± 0.19	19.96 ± 0.09	19.96 ± 0.09	2.49 ± 0.17
	Roasted	6.83 ± 0.40	19.61 ± 0.26	19.61 ± 0.26	0.69 ± 0.25
	Mean difference	0.5	0.35	0.35	1.8
	Probability	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$
Experimental diet	Fresh	4.94 ± 0.17	24.22 ± 0.06	24.53 ± 0.12	10.89 ± 0.35
	Roasted	4.85 ± 0.19	24.53 ± 0.12	24.22 ± 0.06	7.21 ± 0.31
	Mean difference	0.09	0.31	0.31	3.68
	Probability	$p > 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

The results are presented as mean ± SD

After heat treatment, the contents of both fatty acids were lower in control, as well as in experimental samples. The differences were significant ($p < 0.05$) in all cases, except for the stearic acid in samples of meat of pigs fed with experimental diet. Linseed enriched diet positively influenced the content of linoleic acid, and therefore the tenderness of *M. longissimus dorsi* muscle. Previous experiments reported that meat from piglets fed with diet enriched in linseed by adding commercial additives had pleasant color and juicy taste (Okanović *et al.*, 2011).
Roasting of meat caused increase in ω-6/ω-3 as shown in Fig. 1, thus making it less desirable and suitable for human consumption.

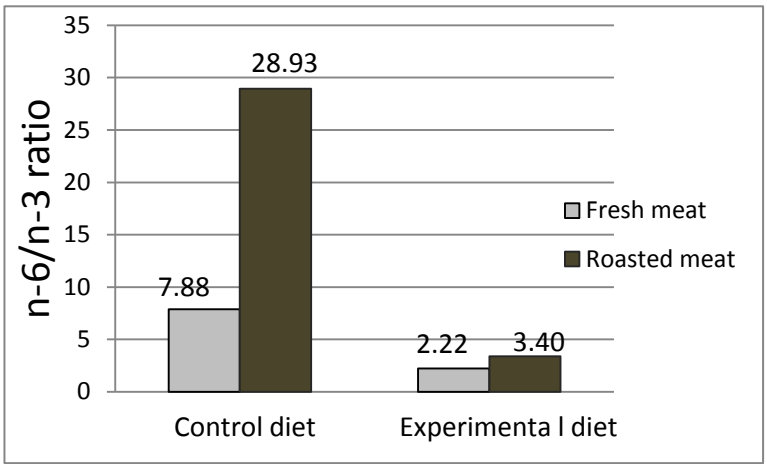

Fig. 1. n-6/ n-3 ration in *M. longissimus dorsi* muscle before and after roasting

Positive effect of linseed diet is reflected in the fact that increase of ω6/ω3 ratio was statistically significant ($p < 0.01$) only for meat samples of pigs fed with control diet. The content of ω-3 fatty acids in control samples was notably low even before roasting, and the heat treatment decreased it below the content of 1%.

CONCLUSION

Presented results have proven that addition of linseed in diet for pigs has multiple benefits considering healthy, nutritional and sensory characteristics of pig meat. Therefore, use of linseed enriched diet in pig feed can be recommended for obtaining meat with desirable fatty acid composition. Enhanced fatty acid composition of fresh meat provides better quality of heat treated meat from the lipid nutrition standpoint.

ACKNOWLEDGEMENTS

This work is a part of Integrated and Interdisciplinary Research Project No. III 46012, funded by the Serbian Ministry of Education and Science.

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TECHNOLOGY OF GERONTO-DIETETICAL SMOOTHIE

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ABSTRACT: Considering the world demographic situation, especially **t**he role of nutritional factors in the pathological aging process, geronto-dietetics has become an independent branch of science. However, food assortments for elderly people are currently insufficient in diversity and amount. Geronto-dietetics production is based on increase in useful substances to the level consistent with physiological consumption rates of the elderly (15-50% of daily requirement). The study of senescence discovers the symptoms of aging which reveal age-related changes in functional systems. For over 100 years the scientists have known about the connection between aging and the state of thyroid gland. The physicians diagnose elderly hypothyreosis at 99%. To prevent hypothyreosis in the elderly one should improve iodine and selenium deficit by increasing food share containing fruit and vegetable sources of selenium and antioxidant vitamins.
Key words: *geronto-dietetics, smoothie, system approach, appetite, elderly, nutritional correction*

INTRODUCTION

Modern ideas about creating new generations of geronto-dietetical foods can be classified as follows: first – nutritionally balanced meals which completely correspond to physiological needs of the elderly; second – food products for dietary correction of nutrition (this area implies processing of foods enriched with one or more ingredienta); third – nutrient premixes which can correct a meal or a daily intake; fourth – producing foods enriched with biologically active components which fortify definite nutritive properties of a product (this category of foods is widespread in economically developed countries); fifth – foods which conduce to prevention of hypothyreosis development (Yudina S.B., 2009).
The necessity of producing such food products is based on the special dietary requirements of elderly people and focuses on specialized nutrition support.
When producing foods for the elderly it is quite challenging to achieve chemical equilibrium only at the expense of their natural reserves. The most promising solution to the problem is to achieve the benefits of several areas simultaneously (Petrov A.N. at al., 2009).
Clinical and physiological studies of human bodies as they age have discovered a number of aging symptoms which reveal age-related changes in functional systems. For over 100 years the scientists have been aware of the connection between aging and the state of thyroid gland. The physicians diagnose elderly hypothyreosis prevalence at 99% (Culicova V.V., 2007)**.** Hypothyreosis or hypofunction of thyroid gland is the disease associated with insufficient supply of organs and tissues with hormones of thyroid gland. Primary hypothyreosis starts because of congenital anomalies of thyroid gland, inflammation due to infectional processes, as complication after surgical operations and radioactive iodine therapy, and as a result of lack of iodine in the environment. The main symptoms of primary hypothyreosis are: weakness, inertia, excessive sleepiness, memory degradation, indifference, absence of interest to surroundings, decrease of capacity for work. The patients suffering from hypothyreosis have inhibited speech, diminished hearing, low hoarse voice. They are lethargic, eye expression is indifferent. Typical symptom of hypothyreosis is edema

of hypodermic cellular tissue which has its own peculiarity: pressing edematous tissues does not leave any fossula (Fadeev V.V., 2004).
The phenomenon is stipulated by complicated disfunctioning of numerous regulation systems of the body which are influenced by a complex of social and economic factors. Only for the last few decades this problem has got a lot of attention in the aspect of pharmacological correction, quite less in applying healthy diet for disease prevention.
To prevent hypothyreosis development in the elderly, besides obligatory pharmacological correction, iodine and selenium deficit should be improved by increasing food share containing fruit and vegetable sources of selenium and antioxidant vitamins, and also hydrobionts which are sources of iodine.

MATERIAL AND METHODS

The ingredients which contain dietary fibers, crucial vitamins and microelements (banana, orange, bilberry, sweet cherry, celery, carrot, sweet pepper, parsley), particularly ground seeds (ground pumpkin seeds, ground flax seeds, ground milk thistle seeds) and non-traditional vegetable oils, have been selected to engineer a smoothie with a polynutrient composition – a gerodietical product.
Swelling analyses of ground milk thistle seeds in tomato juice and ground flax seeds in kefir have been carried out at 18 °C and hydro modules (HM) 1:10, 1:15, 1:20. The mass of functional raw materials used in the experiment is 1.0 g. The swelling index has been estimated for 2 hours. The swelling rate analysis of ground flax seeds in kefir have been carried out on the same conditions as for ground milk thistle seeds in tomato juice.
The viscosity analyses of a “banana – orange” fruit purée with ground pumpkin seeds and a “banana – bilberry – sweet cherry” fruit purée with ground milk thistle seeds being added have been carried out for the samples with ground seeds content of 2…10%.

RESULTS AND DISCUSSION

The aim of the present study is to analyze process variables of ground seeds and model systems “fruit or vegetable purée – ground seeds” for engineering the technology of definite assignment smoothie production.
The ground seeds have already proved to be the nutritional ingredients which enrich foods with minerals, vitamins, dietary fibers, polyunsaturated fatty acids, microelements (zinc, chromium, manganese, selenium) and prevent diseases development.
The chemical compositions of five different ground seeds items produced by Scientific and Production Company “Zhytomyrbioprodukt” have been studied in the experimental laboratory at Ukrainian Institute of Livestock Breeding of the Steppe Regions. According to the obtained results, the ground seeds which contain the highest cellulose content, antioxidant vitamins and mineral substances are ground milk thistle seeds, ground flax seeds, and ground pumpkin ones. They have been selected for further studies.
Swelling indices of ground seeds and viscosity of model systems “ fruit or vegetable purée – ground seeds” have been analyzed to substantiate technological characteristics of swelling. Swelling of vegetable polysaccharides depends on the nature of a solution. According to the food intake monitoring, there is deficiency in vitamin A1, β-Carotene, and calcium in elderly diet. Because of this cultured milk food (kefir) and tomato juice have been used to dissolve vegetable smoothies.
Figure 1 reflects the data which show that the swelling speed of the mixture “milk thistle seeds and tomato juice” has been remaining intensive for 45-60 minutes and comprises 550-560% ground seeds weight per a sample at HM 1:10, 558-570% at HM 1:15, and 563-578 % at HM 1:20 accordingly. The swelling speed of the mixture “ground flax seeds and kefir” (the analysis has been carried out at 18 °C) has been remaining intensive for 45-60 minutes and comprises 710-715 % ground seeds weight per a sample at HM 1:10, 725-730 % at HM 1:15, and 740-745 % at HM accordingly (Figure 2). According to our study, when swelling

ground flax seeds keep moisture twice as much as ground milk thistle seeds and three times as much as ground pumpkin seeds do.

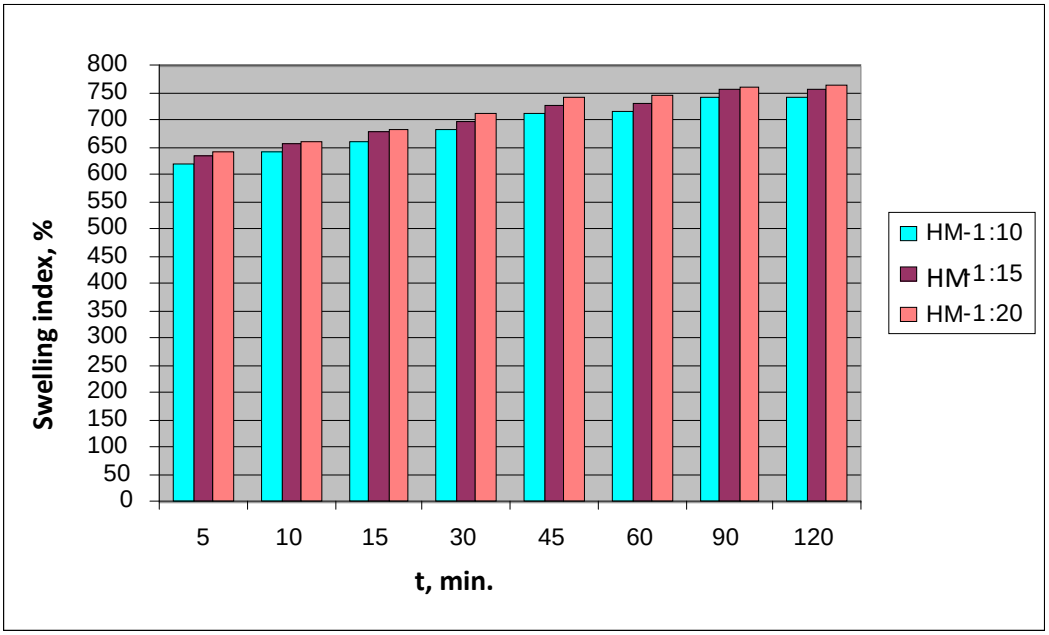

Figure 1. Dependence of swelling index of ground milk thistle seeds in tomato juice on hydro module and time

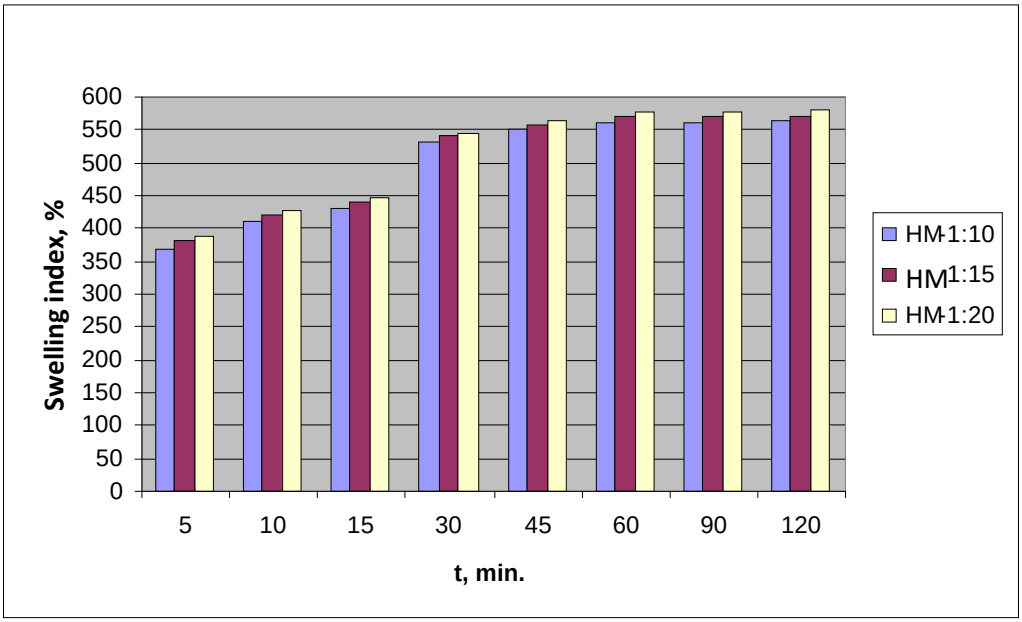

Figure 2. Dependence of swelling index of ground flax seeds kefir on hydro module and **time**

The swelling process stopped after 45-55 minutes. After this time the swollen ground seeds didn't practically change their weight.

According to the results, the most optimal viscosity of model systems is that with 2% ground pumpkin seeds and 4% ground milk thistle seeds.

The results of viscosity studies of "banana – orange – ground pumpkin seeds" mixture and "sweet cherry – bilberry – banana – ground milk thistle seeds" mixtures which contain 2…10% ground seeds are in Fig. 3 and Fig. 4. According to the obtained results, the most

optimal viscosity of model systems is that with 2% ground pumpkin seeds and 4% ground milk thistle seeds. When being defrosted cherries and bilberries exudes enough liquid to make cellulose ground milk thistle seeds swell without worsening thickness and taste properties of a smoothie.

The results of viscosity studies of "celery – carrot" and "carrot – sweet pepper – parsley" vegetable purées with ground flax seeds and with the addition of ground milk thistle seeds have been carried out for the samples with a ground seeds concentration of 2%. The obtained data show that viscosity of a swelling mixture (at 18°C) changes intensively. Then the process is slower but the densest systems are formed by model mixtures which contain ground milk thistle seeds from 6% to 10%. It means that increases in viscosity of vegetable model systems with ground milk thistle seeds and ground flax seeds have the same tendency. Dense systems form model mixtures which contain ground seeds from 6% to 10%.

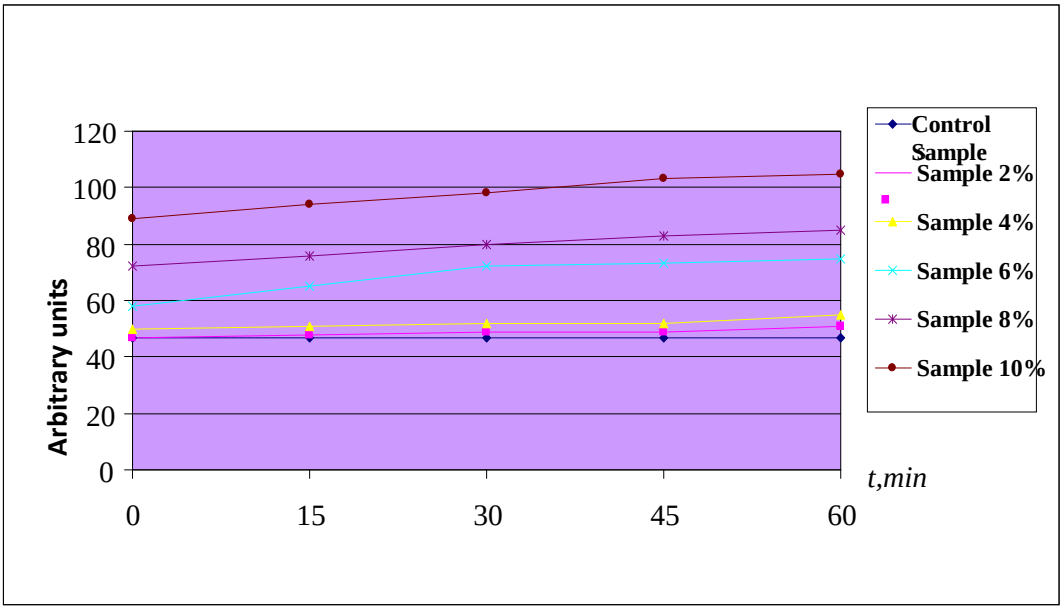

Figure 3. Viscosity analysis of a "banana – orange" fruit purée with ground pumpkin seeds dependent on time for swelling

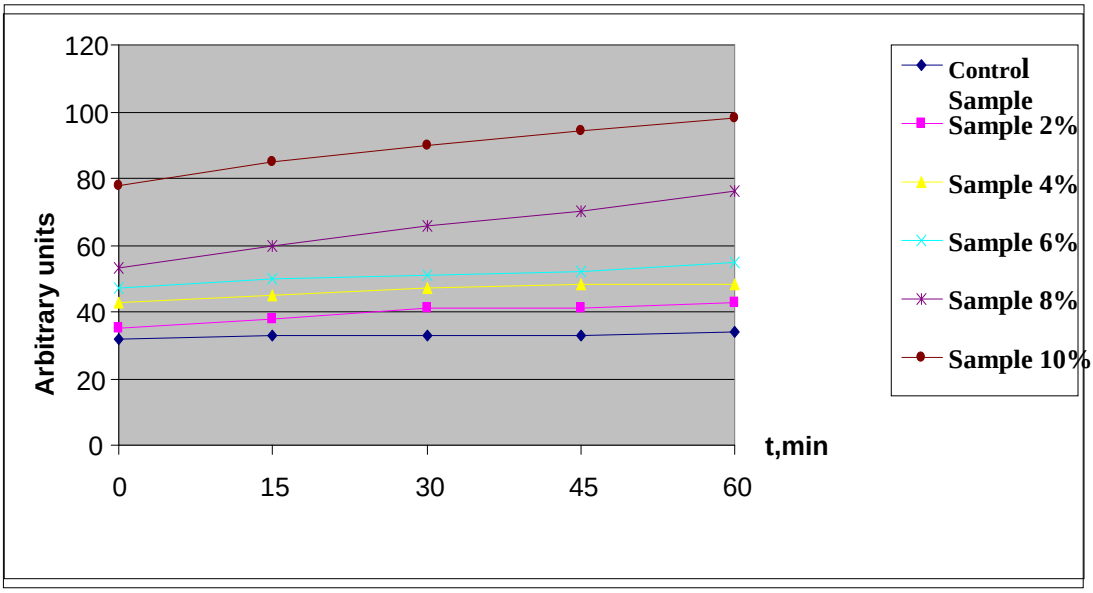

Figure 4. Viscosity analysis of a "banana – bilberry – sweet cherry" fruit purée with ground milk thistle seeds dependent on time for swelling

A system approach to smoothie making technology is represented as an integral system within which fundamental systems – C_1, C_2 and subsystems A, B are singled out. Their functioning is aimed at obtaining the initial result of system operation – engineering gerodietetical smoothies. (Fig. 5).

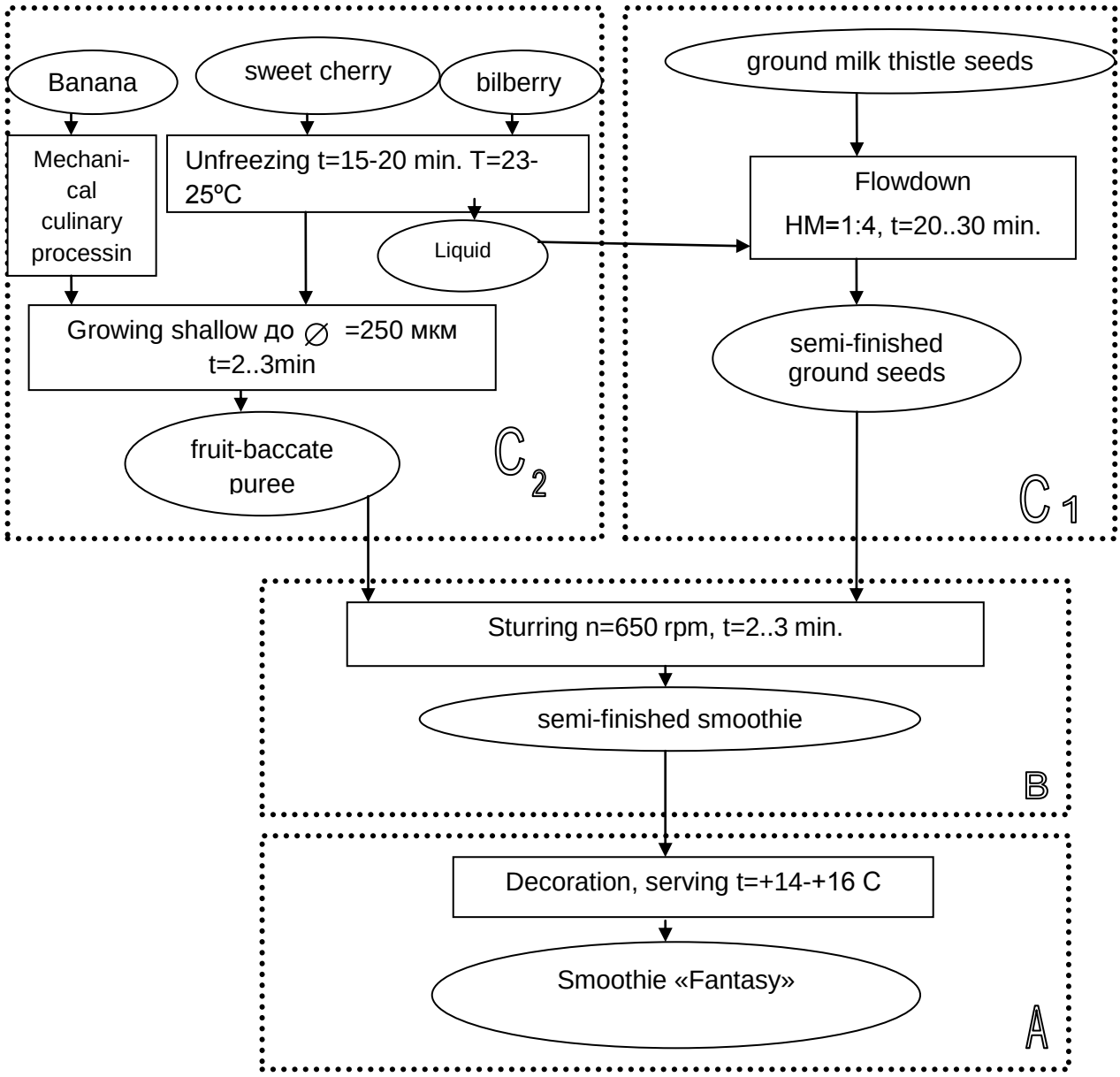

Figure 5. Engineering scheme of Vegetable Smoothie «Fantasy»

CONCLUSIONS

The vegetable, fruit and berry geronto-dietetical smoothie production has been engineered to prevent nutritive hypothyreosis in elderly.The smoothie-gerodietical processing has been designed and engineered on a system approach. The smoothie products which have been studied provide an elderly organism with 40…80% minerals, dietary fibers, and antioxidant vitamins of daily requirement and help it improve.

ACKNOWLEDGEMENTS

This document reflects the ***r***esults of a study that has been carried out within the project «Теоретичне обґрунтування та розробка сучасних конкурентоспроможних технологій смузі-продукції спеціального призначення**»** (Theoretical grounds and development of modern competitive production technologies of special purpose smoothies) financed by the Ministry of Education and Science, Youth and Sports of Ukraine.

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TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF SPRAY-DRIED SOUR CHERRY MARASCA (*Prunus cerasus* var. Marasca) JUICE

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ABSTRACT: Spray drying is an appropriate process for heat sensitive products such as fruit juices rich in polyphenols. However, because of the high sugar content, fruit juices are difficult to dry without carriers and are affected by different drying parameters. Therefore, the influence of two types of malt dextrin (MD) (4-7 dextrose equivalent and 13-17 dextrose equivalent) added in different concentrations (30%, 35% and 40% (w/v)) and inlet air temperature (160, 170 and 180 °C) on total phenols (TP) and antioxidant activity (AOA) of spray-dried sour cherry Marasca juice was studied. Type of malt dextrin used as carrier agent significantly affected the TP content which was higher when using MD 13-17 DE, while there was no significant influence on AOA of powders. The highest TP content was determined in powders with lower concentration of carrier agent, although using MD 13-17 DE in concentration of 35 % resulted in higher content of TP then at concentration of 30 and 40%. Temperature of 160 °C was found to be optimal for spray drying of sour cherry Marasca juice. All studied parameters affected significantly TP content and AOA of sour cherry Marasca powders. The highest content of phenolic compounds was determined in powders obtained using 35 % of MD 13-17 DE at inlet air temperature of 160 °C.
Key words: *sour cherry Marasca, spray-drying, juice powder, total phenols, antioxidant activity*

INTRODUCTION

Sour cherry Marasca (*Prunus cerasus* var. Marasca) is a Croatian autochthonous cultivar characterized with high content of dry matter and sugars, dark red colour and intensive sweet-bitter aroma. It is a very rich source of biologically active compounds, especially anthocyanins which are responsible for its colour. Nevertheless, Marasca cherry contains high concentration of colourless polyphenols which together with anthocyanins contribute to its high antioxidant activity. Due to its high biological value, sour cherry Marasca presents valuable fruit for processing into functional food products.

Spray-drying is a suitable process for drying of heat sensitive products, such as fruit juices rich in biologically active compounds. Spray dryer uses hot air as drying medium which enables the application of high temperatures because of their rapid decrease caused by water evaporation from the product being dried. Therefore, the drying process lasts for a short time, enabling the preservation of valuable compounds present in fruit powders. (Mani et al., 2002). However, fruit powders are natural thermoplastic and hygroscopic properties of fruit juices give rise to problems during the process such as stickiness, caking, etc. (Chegini and Ghobadian, 2007). The stickiness problem is attributed to low molecular weight sugars such as fructose, glucose and sucrose and organic acids such as citric, malic and tartaric acid which constitute more than 90% of the solids in fruit juices (Bhandari et al.,1997). These materials have low glass temperature (Tg) and therefore, are very hygroscopic in amorphous state and loose free flowing nature at high moisture content (Roos and Karel, 1991). Because of the above mentioned, the use of carriers is necessary in order to obtain the free flowing fruit powder, as carrier materials reduce the hygroscopicy by increasing the Tg. Malt dextrin is nowadays one of the common used carriers because of its reasonable price and availableness. Furthermore, it has a beneficial role of an encapsulating agent, increasing the stability of biologically active compounds.

The aim of this study was to study the influence of malt dextrin type, concentration and drying temperature on the content of total phenols and antioxidant activity of sour cherry Marasca juice powders.

MATERIAL AND METHODS

Sour cherry Marasca concentrated juice, produced in 2011, was purchased from "Marasca" factory, Zadar, Croatia. Concentrated juice (65 °Brix) was diluted to 15 °Brix with distilled water. Juice was mixed with malt dextrin (MD) 4-7 DE and 13-17 DE (Sigma Aldrich) added in different concentrations, namely 30, 35 and 40 % (w/v).
Spray drying experiments were conducted on a laboratory scale spray-dryer SD-06A (Labplant, UK) with 1 mm spray nozzle at constant feed rate and air flow, varying the inlet temperature (140 °C, 160 °C, 180 °C). Obtained powders were collected and stored in silica-gel containing desiccators before extraction.
Phenols were extracted from 5 g ± 0.001 powder with 20 mL of 80% aqueous methanol solution containing 0.1 % HCl, in water bath at 60 °C for 20 minutes. Afterwards, extracts were filtered through Whatman No. 40 filter paper (Whatman International Ltd., Kent, UK), transferred in 25 mL volumetric flasks, made up with solvent and used for total phenols and antioxidant activity determinations. All extractions were carried out in duplicate.
Total phenols (TP) were determined by Folin-Ciocalteu method, according to the procedure of Ough and Amarine (1980) with some modifications. The aliquots (250μL) of each extract were added to 25 mL volumetric flasks followed by addition of 15 mL of distilled water, 1.25 mL of Folin-Ciocalteu reagent and 3.75 mL of saturated sodium carbonate and were made up to 25 mL with extraction solvent. The absorbance at 765 nm was measured after tempering for 30 minutes in water bath at 50 ºC. All determinations were carried out in duplicate. The TP content was calculated according to the gallic acid standard calibration curve and expressed as mg of gallic acid equivalents (GAE) per 100 g of powder ± standard deviation (SD).
Antioxidant activity (AOA) was determined using FRAP method as described by Benzie and Strain (1996). Absorbance was measured at 593 nm. All determinations were carried out in duplicate. Results are expressed as mean values mmol of Trolox equivalents (TE) per 100 g of powder.
Analysis of variance (ANOVA) was carried out to determine any significant differences ($p<0.5$) among the applied temperatures, used malt dextrin and their concentrations. The data were analysed using Statistica v. 9 (Statsoft Inc, Tulsa, OK, USA).

RESULTS AND DISCUSSION

The results of TP determination in obtained sour cherry Marasca powders are shown in Table 1. The amount of TP varied from 276.14 mg GAE/100 g in powder produced with 35% MD 4-7 DE at 180 °C to 467.27 mg GAE/100 g in powder produced with 35% MD 13-17 DE at 180 °C. As it can be observed from the results, powders produced with MD 13-17 DE contained significantly higher amount of TP, than those produced with MD 4-7 DE. Statistical analysis also confirmed significant influence ($p<0.05$) of MD type on the content of TP (Table 2.). A malt dextrins characterization study published by Raja et al. (2006) showed that malt dextrins with dextrose equivalent between 10 and 20 fit in the best for use as carriers. Although there was no significant influence of MD concentration and temperature on TP content, it can be seen, that use of higher concentrations of MD 13-17 DE resulted in higher TP content, especially with concentration of 35 %. This can be explained wit role of MD as encapsulating agent, protecting the sensitive ingredients against degradation (Bakowska-Barczak and Kolodziejczyk, 2011). Temperature between 140 and 180 °C did not affect significantly the TP content, although the higest concentrations wer obtained at 160 °C. Kha et al. (2010) also reported that there was no significant difference between temperatures of 140 and 160 °C, and between 160 and 180 °C. The lack of significance may be due to the

drying mechanism during the process. The liquid feed is atomized into a fine droplets in hot air stream, so during the drying step, water evaporation has a cooling effect during the critical drying period. Furthermore, because of the short time of exposure to the high temperatures, sensitive materials are preserved in higher rate when compared to the other drying methods.

Table 1. Total phenols in sour cherry Marasca powders, spray-dried with different carriers added in different concentrations, at three different inlet temperatures

	Source of variation	F_{exp}	p-value
Total phenols	MD type	8,273	0,014*
	MD concentration	0,199	0,822
	Temperature	0,383	0,689
Antioxidant activity	MD type	0,873	0,369
	MD concentration	5,069	0,025*
	Temperature	5,783	0,017*

**Results are expressed as mean (n=4) ± standard deviation*

Table 2. Influence of malt dextrin type, concentration and inlet temperature on total phenols and antioxidant activity os sour cherry Marasca powders

Maltdextrin	Concentration %	Temperature °C	TP mg GAE/100 g*
4-7 DE	30	140	360.35 ± 0.75
		160	387.18 ± 0.69
		180	354.72 ± 0.89
	35	140	365.10 ± 0.67
		160	371.52 ± 0.96
		180	276.14 ± 0.54
	40	140	361.00 ± 0.95
		160	356.11 ± 0.63
		180	359.90 ± 0.51
13-17 DE	30	140	335.43 ± 0.74
		160	454.09 ± 0.69
		180	399.88 ± 0.88
	35	140	366.31 ± 0.61
		160	449.38 ± 0.98
		180	467.27 ± 0.72
	40	140	417.27 ± 0.65
		160	429.09 ± 0.77
		180	376.71 ± 0.85

** Significant differences obtained at p<0.05*

Results of AOA determination are shown on Figure 1. Contrary to TP, AOA of obtained powders was significantly influenced by temperature and concentration of added MD, while MD type did not have influence (Table 2.). AOA was higher in powders produced with the lowest concentration of MD (30 %), because those powders had the highest content of fruit solids in total mass. The temperature effect was similar to the one in the TP content, so temperature of 160 °C was the optimal for production of sour cherry Marasca powders with the highest AOA.

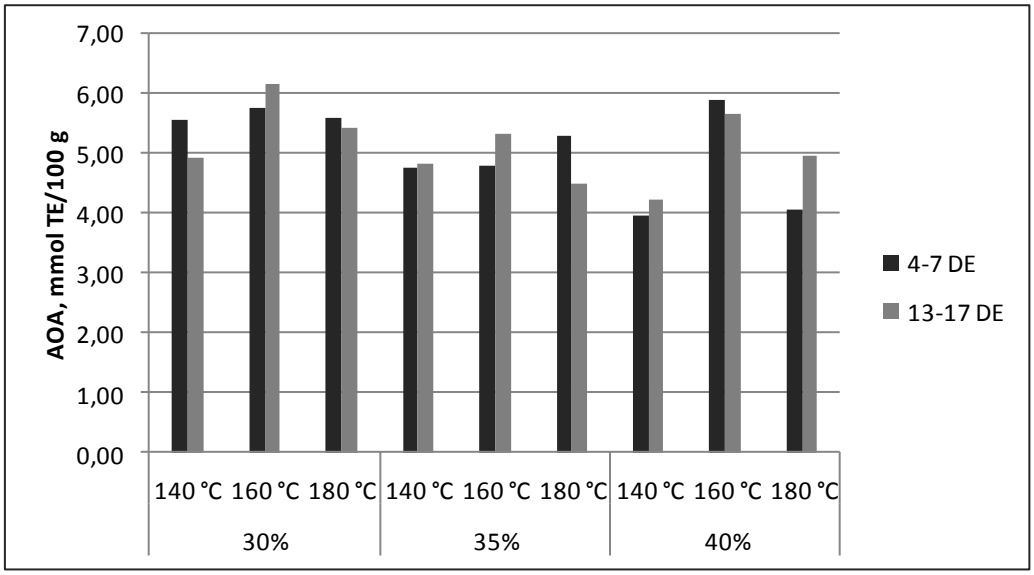

Figure 1. Antioxidant activity (AOA) in sour cherry Marasca powders produced with different carriers added in different concentrations, at three different inlet temperatures

CONCLUSIONS

Type of used malt dextrin significantly affected the total phenolic content of sour cherry Marasca powders, while concentration of added malt dextrin and inlet temperature had significant effect on antioxidant activity. AOA was higher in powders produced with lower concentrations of MD at 160 °C. The highest concentration of total phenols was determined in powder produced with 35 % of MD 13-17 DE at 160 °C.

ACKNOWLEDGMENTS

This paper is a result of the research within the IPA III C project “Marasca Sour Cherry (Prunus cerasus var. Marasca) as an Ingredient for Functional Food”, financed by the European Union.

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BIFIDOGENIC PROPERTIES OF CORN EXTRUDATES

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ABSTRACT: This study determined that the juice of pumpkin, carrot, celery root, parsley, Jerusalem artichoke, and also corn extrudates with their use possess bifidogenic properties. The results indicate that from all types of grain a most bifidogenic effect is possessed by grain of corn after extruding.
Key words: *bifidobacteria, cereal foods, pumpkin, carrot, celery root, parsley, Jerusalem artichoke, bifidogenic factors.*

INTRODUCTION

As early as 2002, Milner (2002) forecasted, that in the coming decades the stake of functional foods could make about 30% of all the food market and could replace many traditional medicinal preparations by 35-50%. Today specialized departments of most food supermarkets sell functional foods. The level of consumption of such products has grown substantially, especially in countries with a developed economy. Depending on the method of receipt, most functional foods are from two basic groups: products from sour-milk and from corn. The corn products such as quick-cooking dry breakfasts, obtained by extrusion, are most common.

The utility of the extruded corn products is not only in higher nutritional value but in a prebiotic effect, which strengthens growth of bifidobacteria (Otles and Cagindi, 2006). Prebiotic properties give food stuffs by creation of effective compositions or by the special technological ways. It acquires large popularity among the producers of products of the functional foods (Ohr, 2004).

Bifidoflora is the basic group of bacteria of the human alimentary tract (Ishibashi et al., 1997). In the organism of a healthy man, the basis of microbiota composition of large intestine is made by bifidobacteria, although in course of time, and also at different diseases the amount of bifidobacteria sharply diminishes and increases the risk of disease disbacterioses (Mitsuoka, 1990). Support of the development of bifidobacteria by the prebiotic factors of basic food stuffs is an important stage of prevention of different diseases. Bifidobacteria produce bioactive substances that regulate many functions of organisms: the work of human gastrointestinal tract, metabolic processes, the work of liver, heart vascular system, blood producing organs, cholesterol reduction, anticancer activity and other health benefits. They promote a vitamin production function – take part in synthesis and suction of vitamins of group B, folic and nicotine acids, in the synthesis of essential amino acids, the best mastering of ions of calcium, iron, vitamin D, own antianaemic, antirachitis, and another actions (Kantha, 1999).

The application of extrusion opened new prospects of enriching of corn products with bioactive substances, which garden- and green-stuff are rich in. It is allows substantially to extend the assortment of functional products on the basis of the cereals. Above all things such green-stuffs, as carrot, pumpkin, Jerusalem artichoke, celery root, and parsley, deserve attention, because of their bifidogenic properties discovered in previous research investigations by many authors (Masanori and Zenzo, 1971).

The purpose of our research was to determine the effect of extrusion of different cereal grains and green-stuff on bifidogenic properties of cereal extrudates.

MATERIAL AND METHODS

Research of bifidogenic properties of samples was carried out by growing of collection cultures of Bifidobacterium adolescentis, Bifidobacterium bifidum, and Bifidobacterium longum in a corn-lactose nutrition medium (reference). The extracts of green-stuff and finely divided samples of cereal extrudates were added to the culture medium after sterilization. After cultivation for 24 - 48 hours at 37 °C, probiotic strains were inoculated in thioglycolic semi-fluid culture medium. Survival of different probiotic strains was monitored by the standard plate count method. Reference cultures of Bifidobacterium adolescentis, Bifidobacterium bifidum and Bifidobacterium longum were grown using a culture medium, which contained 20% more corn extract than the standard medium.

RESULTS AND DISCUSSION

In the first stage of this investigation bifidogenic properties of the extruded grain without additives were studied (Figure 1).

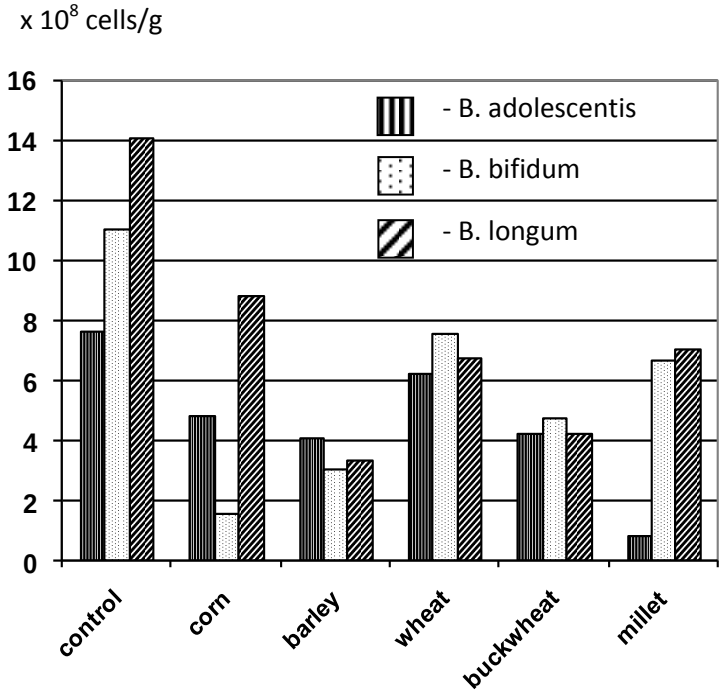

Figure 1.The influence of addition of extruded cereals to a nutrition medium on growth of bifidobacteria

Samples of the extruded corn, barley without a husk, wheat, buckwheat, and millet were added to the nutrition medium. As results in Figure 1 show, none of tested grain samples exceeded the reference. These results indicate that there was insufficient amount of nutrients and bioactive compounds necessary for the successful development and growth of bifidobacteria. There was a different intensity of growth of cultures of B. adolescentis, B. bifidum B. longum with the same composition of the nutrition medium. It is important to note that B. longum grew the best of all bifidobacteria in the controlled culture broth and also after addition of the extruded corn grain the culture broth. By adding of extruded buckwheat to the nutrition medium, as well as wheat and barley without husks, a difference in intensity of cultures growth of Bifidobacterium group was not observed.

The lowest results were marked at adding to the nutrition medium of the extruded millet at growing of culture of B. adolescentis and the extruded grain of corn at growing of culture of B. bifidum.
The next stage of this work was conducted by adding of extrudates of mixtures of grain and green-stuff to the culture broth. They were obtained by the extrusion of mixture of cleared grain and 10-30 % of the cleared grain and the grounded green-stuff. Results from this stage are presented on the Figure 2.

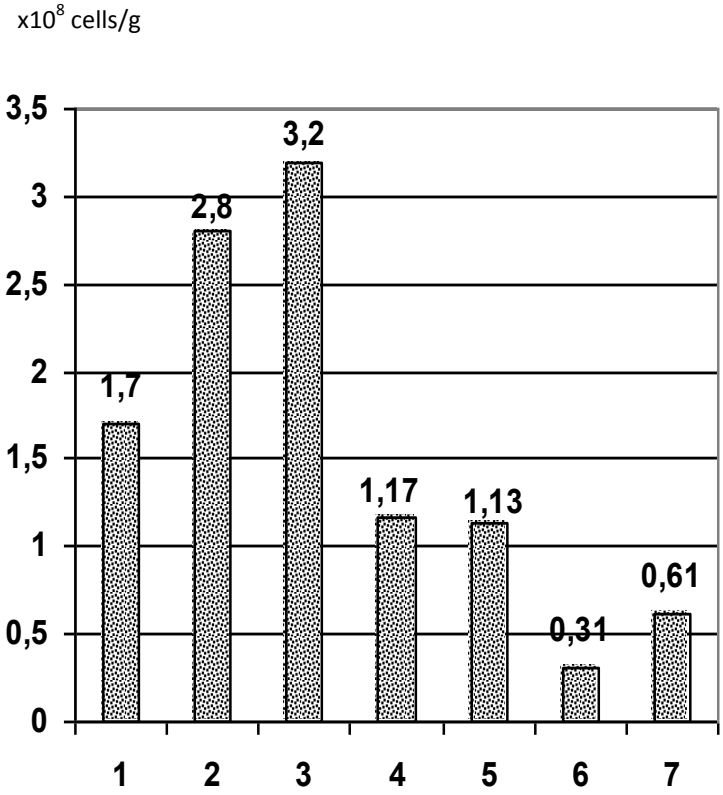

Figure 2. The Influence of addition some products with carrot to a nutrition medium on growth of bifidobacteria: 1- reference; 2- juice of carrot; 3- corn+carrot; 4- barley+carrot; 5- wheat+carrot; 6- buckwheat+carrot; 7- millet+carrot.

Apparently, the addition of 20 % of carrot juice to the nutrition medium increased the amount of B. adolescentis cells by 1.65 times compared to the reference. The addition of the raw carrots to corn resulted in 1.88 fold increase of B. adolescentis cells compared to the reference. In the case of all other combinations of cereals with carrot, the growth of bifidobacteria was slower than in the reference. Similar results were observed in the study with the samples of extruded buckwheat and millet, to which raw carrots were added. They were 5.48 and 2.78 times lower than in the reference, respectively.
Adding pumpkin to grain samples that were subjected to extrusion affected the development of B. adolescentis culture ((Figure 3). The addition of 20 % pumpkin juice to the culture medium resulted in 1.45 fold B. adolescentis growth increase of compared to the reference. The best result was observed by adding extruded mixture of corn grain and pumpkin to the nutrition medium (2.1 times more than reference). Mixing barley and pumpkin during extrusion promoted the growth of B. adolescentis culture in the same way as the reference medium. The lowest results (5.5.and.2.75 times less than reference) were obtained again when pumpkin was mixed with buckwheat or millet, therefore, allowing us to exclude them from subsequent studies.

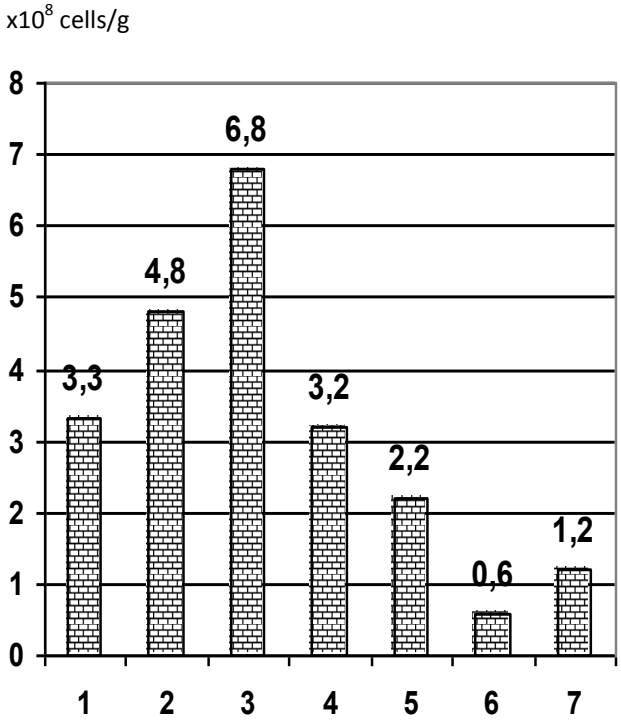

Figure 3.The influence of addition some products with pumpkin to a nutrition medium on growth of bifidobacteria: 1- control; 2- juice of pumpkin; 3- corn+ pumpkin; 4- barley+ pumpkin; 5- wheat+ pumpkin; 6- buckwheat+ pumpkin; 7- millet+ pumpkin

The data in Figure 4 show the results of the effect of celery juice on the growth of bifidobacteria. From the results in Figure 4, it is evident that adding celery root juice to the nutrition medium improved the cells growth of culture of B. adolescentis by 1.72 times in comparison with the reference. A better result was obtained by adding medium samples of the extruded mixture of corn and celery root to the nutrition medium (2.1 times more cells of B.adolescentis than the reference).

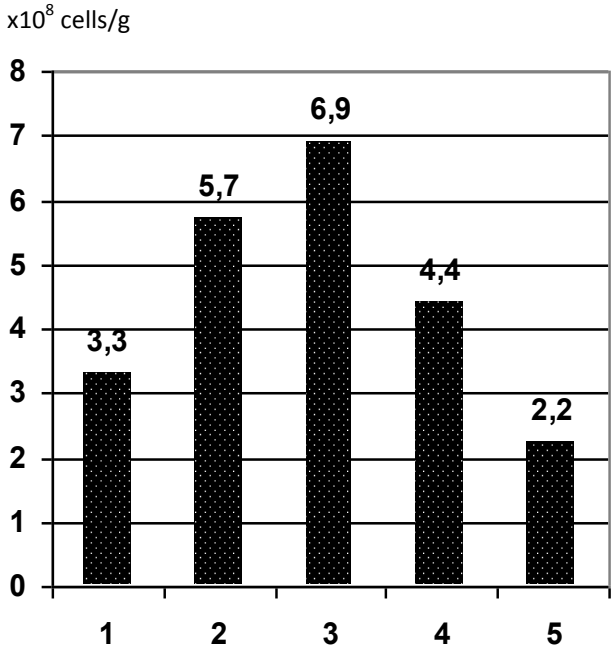

Figure.4. Influence of addition some products with roots of celery to a nutrition medium on growth of bifidobacteria: 1- control; 2- celery juice ; 3- corn+ celery; 4- barley+ celery; 5- wheat+ celery.

A higher number than the reference was also obtained by adding extruded mixture of barley and celery root to the nutrition medium (1.33 times more than the reference). Our previous experiments conducted with extruded mixtures of corn grain, Jerusalem artichoke and/or parsley root suggested that extruding of such mixtures could substantially improve bifidogenic properties of the samples (Figures 5 and 6). For example, by adding extruded mixture of corn and Jerusalem artichoke to the nutrition medium the growth of B. adolescentis culture was 1.86 greater than the reference.

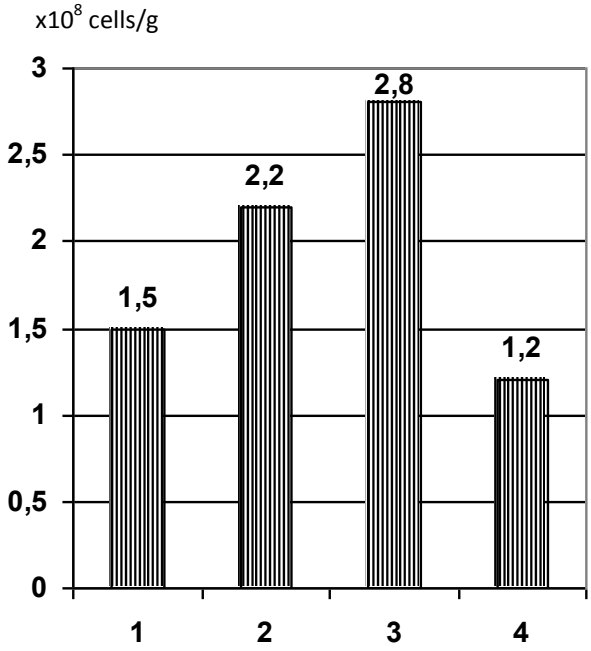

Figure 5. The influence of addition some products with J.artichoke to nutrition medium on growth of bifidobacteria: 1- control; 2- juce of J.artichoke; 3- corn+J.artichoke; 4- barley+ J.artichoke.

The results with parsley root juice alone (Figure 6) confirm its high bifidogenic effect (2.2 times higher than reference). Extruding of parsley root mixed with corn or barley did not have positive effect on the growth of bifidobacteria.

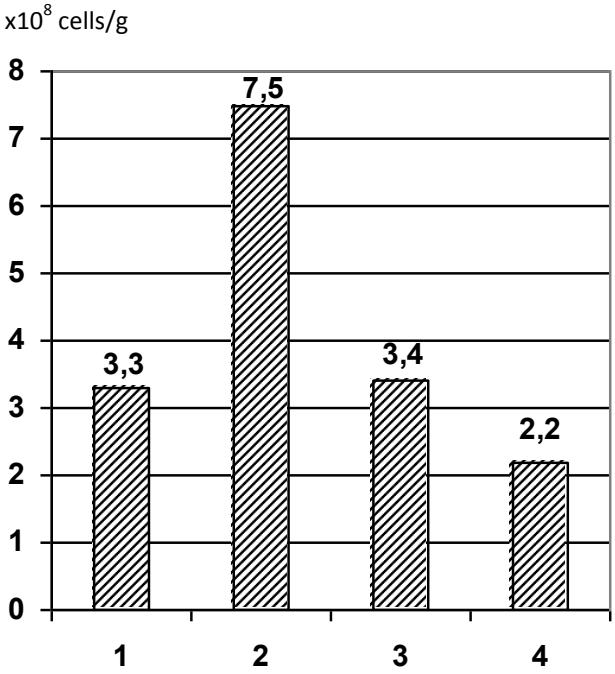

Figure 6. Influence of addition some products with roots of parsley to a nutrient medium on growth of bifidobacteria: 1- control; 2- parsley juice; 3- corn+ parsley; 4- barley+ parsley.

CONCLUSIONS

This investigation demonstrated that extrusion of corn grain and green-stuff could substantially improve of bifidogenic properties of dry corn breakfasts, especially by extruding mixtures of corn with carrots, pumpkin, Jerusalem artichoke, and celery root, as well as, by extruding mixtures of barley and celery root.

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DEVELOPMENT OF PRODUCTION PRINCIPLES FOR FUNCTIONAL FOODS

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ABSTRACT: A model for the development of consumer-oriented properties for new functional foods was developed. This model contains a procedure for the development of a new product and considers the ways to promote a culture of foods consumption as well as consumer development in terms of improved knowledge of nutrition and healthy lifestyle, thus helping a consumer to lay down his requirements accurately and eventually become a full participant in the development of new competitive and high-quality functional food.
Key words: *consumer, consumer properties, model, functional food*

INTRODUCTION

One of the biggest challenges to improve the food patterns of the population is the increase in production of mass-consumption foods of a high nutritional and biological value. Modern food must not only meet the physiological needs of a human body in nutritional, biologically active substances (BAS), and energy, but also to perform preventive functions and, of course, be absolutely safe.
The solution to these problems is possible through the production development of a new generation of foods – functional foods, which meet the requirements and realities of today's life. Ever growing army of supporters of a healthy diet encourages manufacturers to develop a new foods, enriched with vitamins, minerals, dietary fibers, beneficiall bacteria and other nutritional additives. The consumer now believes that "food should not only be delicious, but useful as well" and the statement that was made by Hippocrates some 2.5 thousand years ago "Let food be thy medicine and medicine be thy food" is relevant today more than ever.
Currently, each food producer, which is expecting to take a strong position in the market, should pay great attention to the principles of healthy eating. This is due to the fact that a competition in a food production is very high and the consumer is always making his choice between similar foods. It is simply not enough to produce a new product and introduce a successful advertising campaign, as the product has to offer an extra value that would encourage consumers to buy this particular product. We can say that one of the main principles of modern development of the food industry is the development of new functional foods, contributing to the improvement and preservation of health.
To create a new type of functional food capable of satisfying the customer requirements, it is necessary to develop and produce a food with improved attributes, taking into account all possible factors influencing the consumer while selecting a food. Existing models of development of functional foods are mostly passive, which is complicating the promotion and sale of new foods in the market (Belinskaya S.E., 2010, Minko, E.V. et al, 2005). We have introduced an active model for the development of consumer-oriented properties for new functional foods, which is shown in Figure 1.

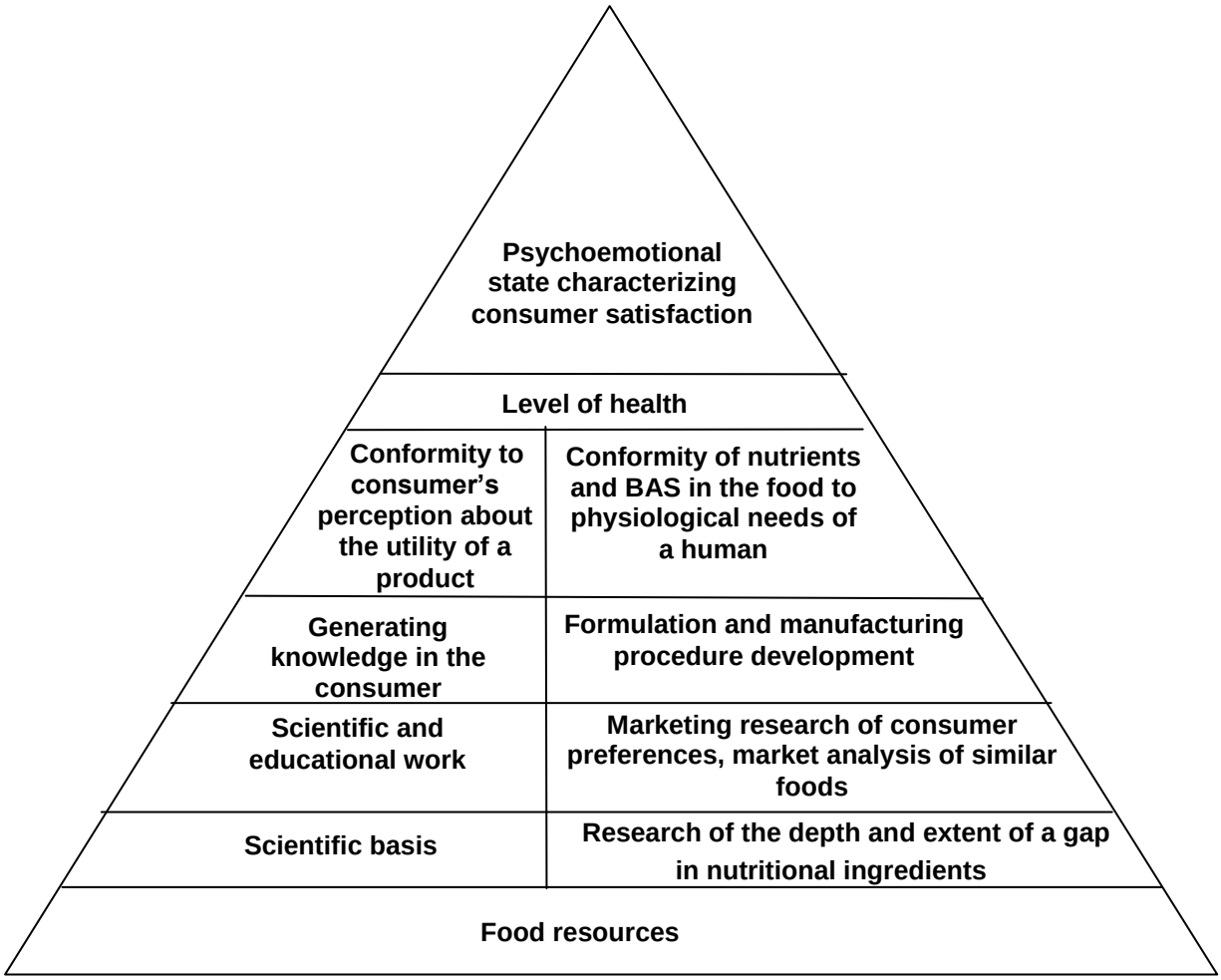

Figure 1. Model for the development of consumer-oriented properties for new functional foods

RESULTS AND DISCUSSION

The basis of the developed model are food resources as the development of functional foods is based on a high quality food economy.

Development of functional foods should start with monitoring the current level of nutrition and health in a population as well as studying the reasons for emersion and extent of shortage of some nutrients. These results are the basis for selecting additional nutrients and BAS, to be introduced into the product, and their amount.

Consideration of the consumer's requirements, desires and behavior is critical to the success of the new food in the food industry (Earl M. et al, 2007). To turn the concept of food into a new product we need a scientific basis in the area of quality of raw materials, enriching additives, manufacturing procedures, physiology of nutrition, "consumer-product" relations, knowledge of marketing, etc. All these disciplines are interconnected as the knowledge of qualitative variables of raw materials effect the knowledge of processing technology. It is important that the developer (producer) considers not only the knowledge in some specific area at the development of a new product but uses a complex approach based on the knowledge obtained from different areas of science. We can say that the development of a product is a knowledge-intensive process and definition of new ideas and concepts requires for a deep knowledge of both the product and the consumer (Earl M. et al, 2010). The consumers may be willing to have a liquid product sold in bottles while the technology may provide for cardboard packaging only, so you need to know how consumers will react to a

cardboard packaging and whether they will accept it at all. The consumer's description of a product may be translated into quantitative indicators which is why it is necessary to know the consumers' requirements for the properties of a product and possess certain methods of measuring those properties. Information like that may be acquired through a marketing research of both existing and potential consumers. This stage is used to define consumer preferences, possible consumption of a new product, and primary and secondary sources of essential nutrients to influence the selection of additives and their amount. If the developer (producer) is focused on the needs of the market, consumers will contribute to the development of a product thus becoming a sort of developers. In the meantime, the developer (producer) performs market analysis of similar existing products and raw materials to determine the manufacturing capabilities of the enterprise in terms of developed goods to establish product requirements, which, if satisfied, will contribute to their sale.

Scientific and educational work among the potential consumers should be provided in order to have a successful promotion and sale of a new type of functional food. This work is supposed to develop knowledge on the dangers and benefits of certain nutrients and BAS as well as their standards of consumption and contents in food raw materials and products among the consumers. Based on this information is the development of the consumers themselves, resulting in a change of their level of knowledge, which influences the decision on whether to buy a product or not. We have created a model for the development of consumer preferences with the consumers, depending on the knowledge they've received. Under this model, the consumers may be subdivided into 3 levels according to their level of knowledge (Figure 2). A consumer with a low level of knowledge is buying a product while

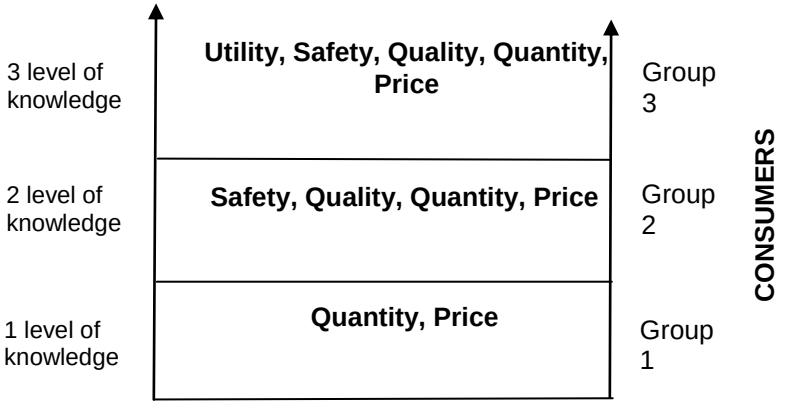

Figure 2 Model for the development of consumer preferences depending on the knowledge they've received

paying major attention to his knowledge on the cost and relying primarily on the material side of the issue, i.e. price, quantity, and their relationship are considered while buying a product.

As the level of consumers' knowledge on nutrition and healthy lifestyle grows, they start to consider the safety and quality of the product besides the price. Consumers with high level of knowledge will turn their attention to functional or organic food, i.e. they'll be primarily concerned with such criteria as utility, quality, natural ingredients, and safety while looking at the cost, quantity, and their relationship after all the others. The result of scientific and educational work among the population is the evolution of consumer in terms of nutrition, healthy lifestyle, and ability to lay down his requirements while ultimately influencing a decision on whether to buy a new product, development of consumer preferences, and consumer demand.

The purpose of pre-production stage in the life cycle of a functional product is to develop a formulation and technology of its production with the subsequent release of pilot batch. This stage is the most important in developing of a functional food as this is when the quality and consumer-oriented properties (including functional) of a new product are shaped, which largely determine its competitiveness and demand with the consumers. Design and development of new formulation for a functional product should provide for assimilability and

effectiveness of enriching additives as well as their safety. The purpose of this stage is to maximize compliance of developed product with the quality requirements.
The second stage in the life cycle of a product is a specific set of actions taken to ensure a production of high quality food and its sale on the market. All activities related to a production of food with improved consumer-oriented properties should be planned at the design stage and provide for an introduction of high-performance management in compliance with ISO, use of modern manufacturing procedure with consideration to operating conditions, high efficiency of equipment handling, quality of personnel performance, etc. (Earl M. et al, 2010). Manufacturing procedure (conventional or modified), apart from providing consistent quality and safety of a product, has to ensure maximum shelf life of enriching additives in the process of production and storage. This stage includes the development and adoption of product manufacturing documentation and obtaining permissions of competent organizations to release the functional product. The release of a pilot batch of the new product and comprehensive quality trade analysis complete the production phase allowing for further steps to be made (manufacturing application). Since the developed product is positioned as a functional product, a biomedical research should be provided to study the preventive qualities of a new product, i.e. determine the suitability of the contents of food and biologically active substances to the physiological needs of a human body.
It is important not only to develop and manufacture a product with improved consumer properties but to keep these properties during product promotion. In this case, the manufacturer should pay particular attention to the use of high-quality packaging material, compliance with the conditions and requirements for transportation, storage and sale of goods.
The last stage in the development of a functional product is to bring a product to the consumer. It is difficult to overestimate the importance of this stage, as all the previous stages are nothing without it. This is because the developer (producer) of food should not only produce the products with improved consumer properties, but to develop and implement all necessary measures to bring a new product to the consumer effectively. Market promotion of functional products has its peculiarities – in this case it is not only about marketing development but the encouragement of people to buy a new product as well, it is about maintaining the health of every citizen, regardless of education, age, social status, etc.
The developed model provides for the consideration of selected system – functional product in its dynamics, thus the logical conclusion of a model would be the monitoring of nutrition and health of population. It allows you to evaluate the effectiveness of the system: if the positive result was reached – reduce the content of enriching additives or increase the content of enriching additives and expand the range of functional foods if the deficiency level of essential nutrients was not changed.
When deciding to buy a product that was produced with consideration to all activities proposed under this model, the consumer gets the product with improved consumer properties. The result – human body is healing and he derives a satisfaction from the purchased product. At the same time, developer (producer) derives satisfaction from developed and manufactured goods as well, while receiving an economic benefit.

CONCLUSIONS

An introduced model for the development of consumer-oriented properties is of an active type. It allows one not only to create a high-quality food product bringing satisfaction to consumer and producer, but, what is more importantly, to establish culture of food consumption, evolution of consumer in terms of nutrition, healthy lifestyle, and ability to lay down his requirements while ultimately influencing the consumer demand that will result in the production of high quality, popular and rival product.

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EXTRACTION OF CAFFEINE AND CHLOROGENIC ACID FROM GREEN COFFEE BEANS AND GUARANA SEEDS

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ABSTRACT: The influence of operating parameters on extraction of caffeine and chlorogenic acid from Green coffee beans and Guarana seeds was studied. Extractions were performed using two different processes, conventional extraction with methanol and supercritical fluid extraction (SFE) with carbon dioxide. Conventional extraction was performed with maceration at solvent boiling temperature. SFE was performed with supercritical carbon dioxide on a semi continuous flow apparatus at pressures 200 and 300 bar, and temperatures of 40, 60 and 80 °C. The raw material, residual material and the extracts were analysed on caffeine and chlorogenic acid content using HPLC method. With conventional extraction both, caffeine and chlorogenic acid, were concentrated in the extract while by SFE with CO_2, the caffeine was concentrated in extract and chlorogenic acid was concentrated in the residual material. The highest total extraction yield in case of Green coffee beans was obtained at 300 bar and 80 °C and it was 13.8 wt.% and in case of Guarana it was 3.63 wt.%. The content of caffeine from Green coffee beans was the highest at 200 bar and 60 °C where the extract contained 13.44 mg of caffeine/g material and decreases with higher pressure. The content of caffeine from Guarana seeds was the highest at 300 bar and 80°C where the extract contained 9.97 mg of caffeine/g material. The results were compared with those obtained using conventional extraction with methanol where both, caffeine and chlorogenic acid were extracted.
Key words: *extraction, green coffee beans, guarana seeds, caffeine, chlorogenic acid*

INTRODUCTION

Among natural sources of caffeine, including cocoa beans (0.2 wt.%), tea leaves (1.5 – 2.5 wt.%) and coffee beans (0.9 – 2.4 wt.%), guarana seeds are likely the richest vegetable source of caffeine (3 – 6 wt.%) (1). Green coffee beans (*Coffea Arabica*) are raw, unroasted coffee beans with content of caffeine up to 2.4 wt. %. Beside caffeine, green coffee beans also contain nicotinic acid, some proteins, polysaccharides, trigonelline, chlorogenic acid, etc. Roasting is a complex process that causes thermal degradation mainly of chlorogenic acid and trigonelline to phenolic substances. According to literature, during roasting about 30 wt. % of caffeine was lost and the degradation of trigonelline and chlorogenic acid was up to 83 and 95 wt. %, respectively (2). Guarana (*Paullinia cupana*, Sapindaceae) is a Brasilian native plant. Guarana seeds also contain theophylline and theobromine, catechins, proanthocyanidins, phenolic acids, nicotinic acid, taninns and many other components. Because of that, guarana seed extracts are mainly used in concentrates and soft drinks and as ingredients of a variety of pharmaceutical products (3).
The removal of caffeine is usually achieved with organic solvents (as methylene chloride) or with hot water and both can be environmentally harmful. Alternative process is supercritical fluid decaffeination. Supercritical extraction of caffeine from green coffee beans results in undamaged beans, no solvent residue and no colour or flavour losses (4). Supercritical fluid extraction of guarana seeds has been reported by several authors where supercritical CO_2 was used as solvent for decaffeination of seeds (1,3,5). For the process of decaffeination authors were using wet seeds, where the extraction allowed the removal of up to 98 % of initial caffeine content. Extraction of dry seeds is exceedingly slow, as can be also found in this work. Chlorogenic acid was extracted and purificated using organic solvents from plant materials such as coffee beans, apple, potato and other (6).

The aim of this work was to investigate the influence of operating parameters on conventional and supercritical fluid extraction of caffeine and chlorogenic acid from Green coffee beans and Guarana seeds. Supercritical fluid extraction was performed with supercritical carbon dioxide on a semi continuous flow apparatus at pressures 200 and 300 bar, and temperatures of 40, 60 and 80 °C. The constant flow rate of carbon dioxide in range from 0.06 to 0.1 kg/h was maintained during extraction. The raw material, residual material and the extracts were analysed on caffeine and chlorogenic acid content using HPLC method. The results were compared with those obtained using conventional extraction with methanol.

METHODS AND MATERIALS

Material

Solvents for extraction and analysis procedure were purchased from Merck (Darmstadt, Germany). Standard of caffeine (purity 99 %, Cat.No. 2760) was supplied from Fluka (Germany) and chlorogenic acid (purity 99 %, Cat. No. C3878) from Sigma-Aldrich (USA). CO_2 (2.5) was supplied by Messer (Ruše, Slovenia). Seeds from guarana (*Paullinia cupana*, Sapindaceae) and green coffee beans (*Coffea Arabica*) were purchased from a local market.

Analysis

Components in raw material and in extracts were analysed using HPLC analytical methods. For the quantitative determination of chlorogenic acid, HPLC apparatus used was composed of Agilent 1100 Quaternary HPLC pump and Agilent 1100 DAD detector. The column Kromasil C-18 (250 x 4.6 mm, with 5 µm particles) was used. The method was isocratic using 50 mM phosphatic buffer pH 4 /methanol (90/10 v/v) as mobile phase with flow rate 1 mL/min. Extracts were diluted in methanol and analysed at wavelength 314 nm. The same HPLC system was used for quantification of caffeine at 274 nm. The mobile phase water / methanol / acetic acid (5.75/4/0.25 v/v/v) was pumped through Zorbax C-18 (150 x 4.6 mm, with 5 µm particles) column with flow rate 0.5 mL/min. Determination of components in raw material and in residue was performed with extraction procedure at 40°C for 2 h using 1 g of material and 50 mL of methanol. After extraction, the mixture was filtered through 0.45 µm Teflon membrane filter and analysed. The results of analysis were expressed as content of compound per mass of material (Eq. 2).
Each data point represents the average of at least three measurements and the relative standard deviation between measurements was less than 1 %.

Calculations

The results of extraction procedures and analysis were calculated as:

$$extraction\ yield\ (wt.\%) = \frac{m_{extract}\,(g)}{m_{material}\,(g)} \cdot 100 \quad (1)$$

$$content\ (mg/g) = m_{compound}\ / m_{material}\ = \frac{extraction\ yield}{100} \cdot \frac{m_{compound}\ (mg)}{m_{extract}\,(g)} \quad (2)$$

$$extraction\ efficiency\ (wt.\%) = \frac{m_{compound}\ (g)}{m_{extract}\,(g)} \cdot 100 \quad (3)$$

Supercritical fluid extraction (SFE). The SFE experiments using CO_2 were performed on a semi continuous flow apparatus (7). Approximately 15 g of ground material was charged into the extractor (V = 60 mL). The temperature in the water bath was regulated and maintained at constant level (± 0.5 °C, LAUDA Königshofen, Germany). The liquefied gas (CO_2) was continuously pumped with a high pressure pump (ISCO syringe pump, model 260D, Lincoln, Nebraska) through the preheated coil and over the bed of sample in extractor. The solvent

flow rate was measured with a flow meter. The product precipitated in separator (glass trap), where the separation was performed at 1 bar and at room temperature. As result of supercritical extraction the extraction yield (Eq. 1) was calculated.
Conventional extraction. The conventional extraction experiments were performed at boiling temperature using methanol as solvent (10 g of grinded material in 150 mL of solvent). Grinded raw material with defined particle size was extracted using methanol as solvent at mass ratio material: solvent = 1:15. Extractions were carried out for 4 h at boiling temperature, which was for methanol 70 °C. After extraction, the solution was filtered through 0.45 µm filter and solvent was evaporated on vacuum evaporator. Extract was than prepared for analysis with HPLC methods described for determination of caffeine and chlorogenic acid content.

RESULTS AND DISCUSSION

SFE experiments of green coffee beans and guarana seeds were performed using CO_2 as solvent on semi continuous flow apparatus in lab scale. Extractions were performed at pressures 200 and 300 bar and temperatures 40, 60 and 80 °C. The constant flow rate of carbon dioxide in range from 0.06 to 0.1 kg/h was maintained during extraction. The extraction yield and extraction rate increased with increasing pressure from 200 bar to 300 bar at all temperatures investigated.

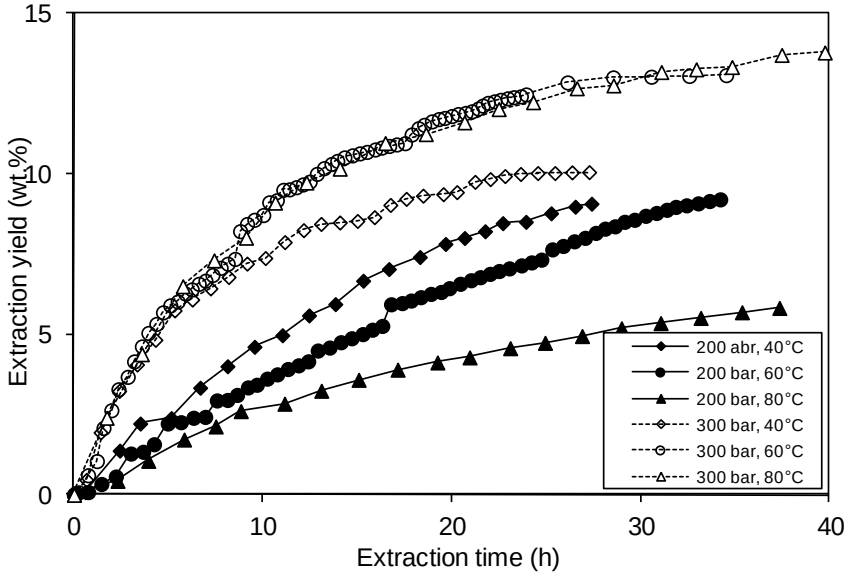

Figure 1. SFE of Green Coffee beans with CO_2.

In case of green coffee beans the highest extraction yield (13.8 wt.%) was obtained at 300 bar and 80 °C. At the same conditions, the highest extraction yield (3.63 wt.%) was obtained for guarana extraction. The extraction curves, shown on Figure 1 and 2 for Green coffee beans and Guarana seeds, respectively, shows that long extraction times are needed to reach constant cumulative extraction yields, to reach full depletion of material.
On Figures 3 and 4, the content of compounds (in mg) per g material (Eq.2) in raw material, methanolic and supercritical extracts, residual material after extraction is presented for Green coffee beans and Guarana seeds, respectively. In raw material, the content of compounds in Green coffee beans was 23.93 mg caffeine/g material and 52.56 mg chlorogenic acid/g material. Guarana seeds contained 46.68 mg caffeine/g material and 8.38 mg chlorogenic acid/g material. Figure 3 shows, that supercritical extracts mainly contained caffeine while chlorogenic acid remained in residual material. Supercritical extracts contain less than 0.1 % of chlorogenic acid due to low solubility in supercritical CO_2. The content of caffeine from

Green coffee beans was the highest at 200 bar and 60 °C where the extract contained 13.44 mg of caffeine/g material and decreases with higher pressure. The content of caffeine from Guarana seeds (Figure 4) was the highest at 300 bar and 80°C where the extract contained 9.97 mg of caffeine/g material. The results were compared with those obtained using conventional extraction with methanol where the content of caffeine was 23.21 and 45.43 mg of caffeine/g material for Green coffee beans and Guarana seeds, respectively. At supercritical extraction, the caffeine was concentrated in supercritical extract, while the chlorogenic acid remained in the residual material.

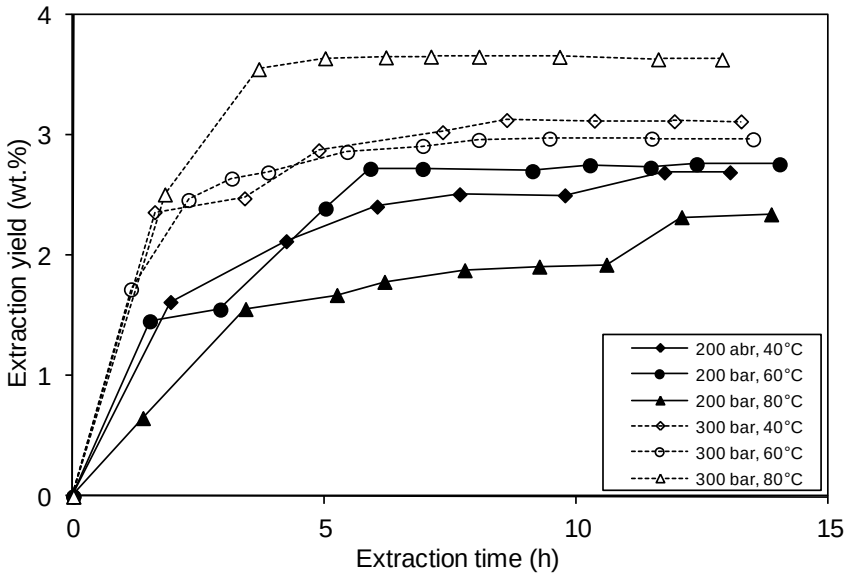

Figure 2. SFE of Guarana seeds with CO_2.

The removal of caffeine using supercritical CO_2 was 56 % and 22 % for Green coffee beans and Guarana seeds, respectively. The raw material used in this research contained less than 5 wt.% of water. According to literature cited (1), authors proposed the usage of wet Guarana seeds for caffeine extraction. The removal of initial caffeine up to 74.7 % from wet Guarana seeds (saturated with water, up to 40 wt.%) was obtained at 70°C and 400 bar. To achieve removal of 97 wt.% of initial caffeine from the wet seeds, also water-saturated CO_2 was used (3).

The extraction efficiency (Eq. 3) of caffeine from Green coffee beans was the highest at 200 bar and 60°C where the extract contained 14.59 wt. % of caffeine. The residual material after extraction at 300 bar and 80 °C was subsequently extracted by conventional extraction procedure and the highest value of chlorogenic acid in extract was 5.15 wt. %. The same results for Guarana seeds extraction were obtained. The extraction efficiency of caffeine from Guarana seeds was the highest at 300 bar and 80 °C where the extract contained 31.75 wt.% of caffeine. Extract obtained with conventional extraction using methanol as a solvent reaches the highest value of chlorogenic acid (2.93 wt.%).

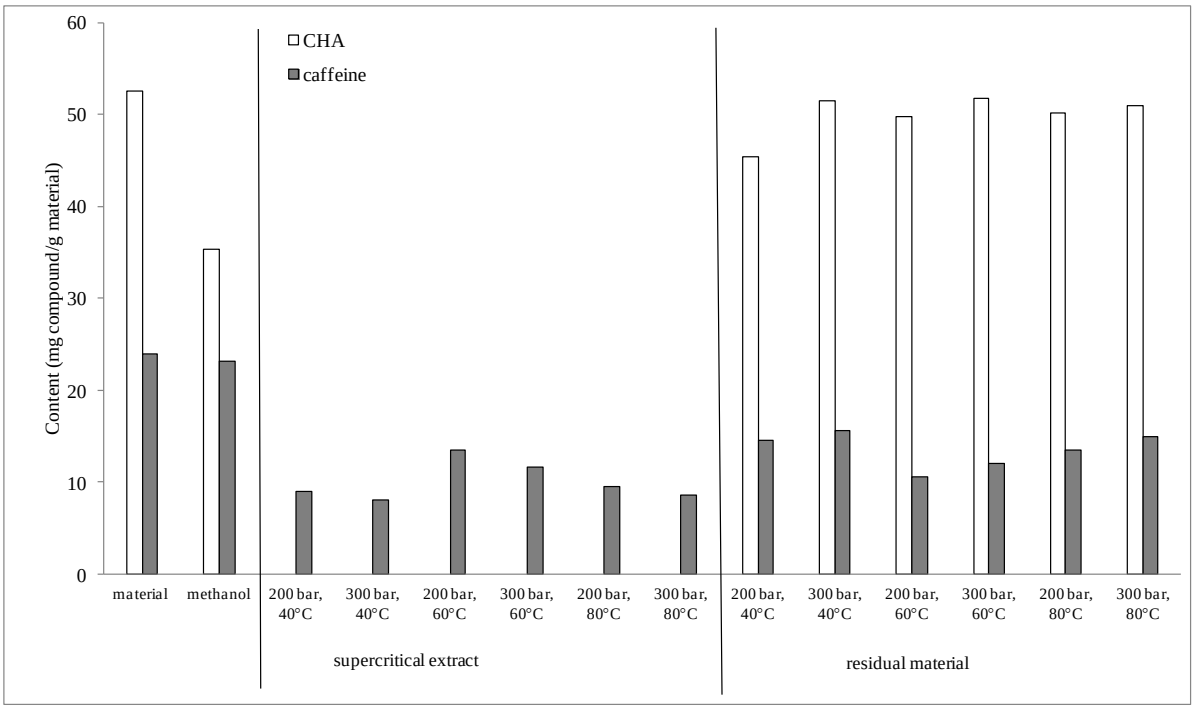

Figure 3. Content (mg compound/g material) of chlorogenic acid (CHA) and caffeine (CAF) in Green Coffee beans after conventional and supercritical extraction.

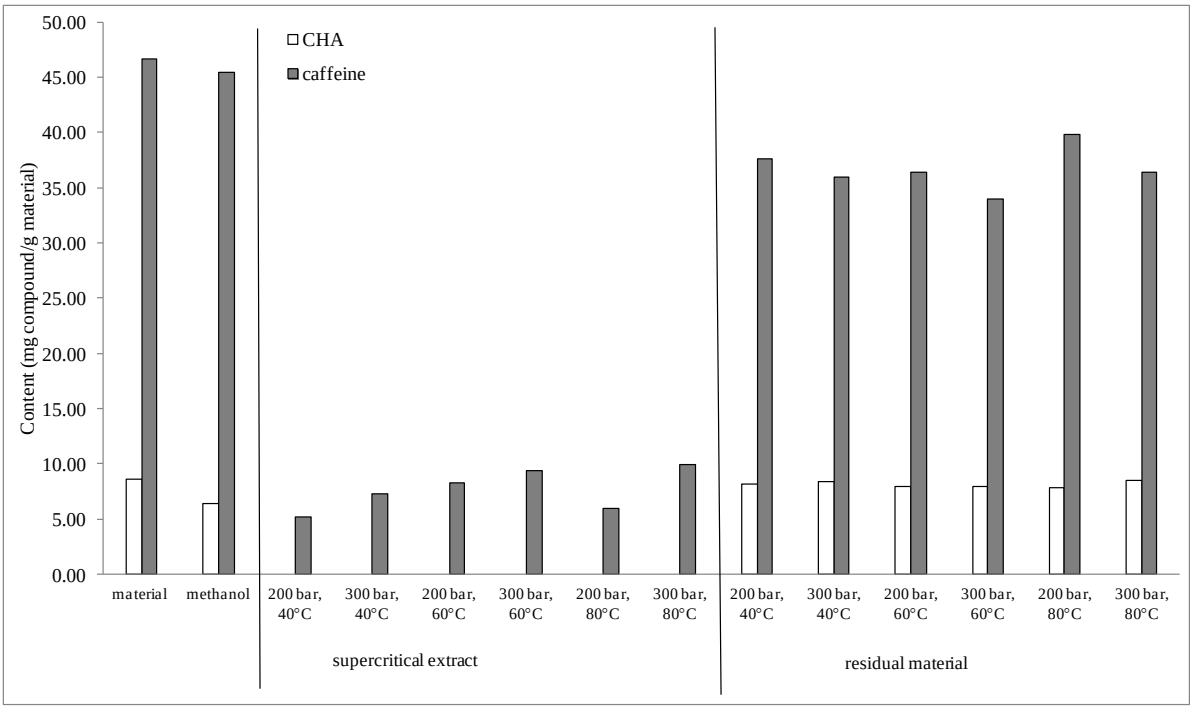

Figure 4. Content (mg compound/g material) of chlorogenic acid (CHA) and caffeine (CAF) in Guarana seeds after conventional and supercritical extraction.

CONCLUSIONS

It can be concluded:

- The best conditions of SFE with CO_2 were 300 bar and 80 °C where the extraction yield reaches 13.8 wt.% for Green coffee beans and 3.63 wt.% for Guarana seeds.
- Extraction yield increased with increasing solvent density and at constant solvent density the extraction yield was increased with increasing temperature.
- The SFE extracts mainly contained caffeine, while the chlorogenic acid remained in the residual material.
- The extraction efficiency of caffeine from Green coffee beans was the highest at 200 bar and 60 °C where the extract contained 14.59 wt. % of caffeine and from Guarana at 300 bar and 80 °C where the extract contained 31.75 wt.% of caffeine.
- Conventional extraction using methanol as solvent gives the highest content of caffeine and chlorogenic acid in extracts, regarding the extraction of both components.
- Removal of caffeine using supercritical CO_2 was 56 % and 22 % for Green coffee beans and Guarana seeds, respectively.

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NUTRITIONAL VALUE OF THE OIL EXTRACTED FROM THE PUMPKIN SEED OIL CAKE

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ABSTRACT: Oil cake is a by-product which remains after the pressing of pumpkin seeds. Until recently, it was mainly used as animal fodder, but lately it has been increasingly used for nutritious food products or ingredients. As the cake retains a considerable portion of oil, the aim of this research was to determine the content of oil in the cakes obtained after pressing 7 samples of naked pumpkin seeds and 3 samples of husk pumpkin seeds, and the nutritional value of the residual oil. The content of oil varied from 11.0 to 16.0 % in dry matter. After that, in the next 24 hours, the oil left behind in the cake was extracted by hexane, at room temperature. The composition and content of fatty acids and the total content of tocopherols were determined. The dominant acids were oleic acid (37.1 – 43.9%) and linoleic acid (30.8 – 44.5%), an ω-6 fatty acid. There was also a small portion (0.1 – 0.3%) of linolenic acid, an ω-3 fatty acid. The total content of tocopherols was considerably high (28.7 – 64.5 mg/100g), with the γ-isomer being the dominant one (73.6 – 85.3% of the total content).
Key words: *pumpkin seed, oil cake, fatty acids, tocopherols*

INTRODUCTION

The utilization of by-products of oil processing is a very important issue for the edible oil industry. Oil cakes, remaining after pressing the seeds, contain considerable amounts of oil, proteins and nutrients, which contribute to the nutritional value of the cake. However, certain kinds also contain antinutrients, which are unacceptable and must be removed in order to ensure the edible status of the cake.

But this is not the case with the pumpkin seed oil cake, a by-product of cold pressing. To date, scientific papers have reported data regarding the use of pumpkin seed oil cake flour in producing cookies, bread and a spread similar to peanut butter (Radočaj et al., 2011).

Since oil, present in a significant percentage in the cake, has not been, so far, a subject of scientific research, the objective of this study was to assess the remained oil by the means of organic solvent extraction, and then estimate the nutritional quality of the oil. To that end, the composition of fatty acids and the total content of tocopherols were determined.

According to the majority of literature data, linoleic (C18:2), oleic (C18:1), palmitic (C16:0) and stearic acid (C18:0) are the most abundant ones. These four acids make up 98% of the total fatty acid content, while the content of other acids does not exceed 0.5% (Murkovic et al., 2004; Nakić et al., 2006; Vujsinović et al., 2010). Fatty acid content may vary depending on diverse factors, such as: the variety of pumpkin, the area of cultivation, weather conditions, stage of ripeness, etc (Griffith et al., 1997; Younis et al., 2000; Siegmund and Murkovic, 2004).

Tocopherols belong to non-glyceride constituents of vegetable oils. Given that they slow down the autoxidation process, their presence is of high importance to the stability of vegetable oils. They have the ability to stabilize free radicals and transform peroxides into stable products, thus ending the chain reaction (Schuler, 1990). Variations in total tocopherol content are relatively high, as a result of the influence of genotype and cultivation conditions,

and the method of oil extraction. However, regardless of these variations in total content, the composition of tocopherols in the pumpkin oil is quite stable. The predominant tocopherol isomers in the pumpkin oil are γ- and α-isomers (Murkovic and Pfannhauser, 2000; Fruhwirth et al., 2003; Gorjanović et al., 2011).

MATERIAL AND METHODS

The starting materials were 7 samples of pumpkin naked seed oil cakes and 3 samples of pumpkin husk seed oil cakes, remained after cold pressing.

Total oil content and moisture content

Contents of oil and moisture were determined according the standard analytical methods, respectively: ISO 659: 2007 and ISO 665: 2008.

Oil extraction

The oil that was left over after cold pressing was extracted from the cake by organic solvent, hexane, at room temperature, over 24 hours. The proportion of cake and solvent was 1:3. After that, the solvent was evaporated in the rotary vacuum evaporator, at the temperature of 40 ^{o}C.

Fatty acid determination

Fatty acids were transformed to fatty acid methyl ester by direct transesterification for the neutral samples and with boron trifluoride solution for samples with higher content of free fatty acids (*ISO 5509,2000).* Fatty acid composition was determined by gas chromatography (GC; VARIAN chromatograph, model 1400; Varian Associates, Walnut Creek, CA), equipped with a flame ionization detector and a 3.0 m × 0.32 cm steel column, packed with LAC-3R-728 (20%; Cambridge Ind. Co., Cambridge, UK) on ChromosorbW/AW(80-100 mesh; Merck, Darmstadt, Germany). Nitrogen was used as a carrier gas (flow rate, 24 mL/min) (*ISO 5508,1990).* Fatty acids were identified by comparison of their retention times (Rt) with those of standards (SupelcoTM FAME Mix). All determinations were carried out in triplicates.

Tocopherol content

Quantification of tocopherols was carried out using high performance liquid chromatography (Waters M600E, USA) on a Nucleosil 50-5 C18 reversed phase column with fluorescence detection and external standard solutions of different tocopherols. The following procedures were applied: n-hexane extraction, extract vaporization and reconstitution in methanol using membrane filtration. Mobile phase was 95% ethanol with a flow rate of 1.2 ml/min. The fluorescence detector (Shimadzu RF-535, Japan) operated with the excitation wavelength at 290 nm and the emission wavelength at 330 nm.

Statistical analysis

Data are reported as means ± SD (n = 3). Statistical analysis was performed using Statistica 7 software package.

RESULTS AND DISCUSSION

Contents of oil and moisture in the samples of cakes remained after pressing the seeds are displayed in Table 1.

Table 1. Amounts of remained oil and moisture content in the samples of cakes

Cake sample	Oil content		Moisture content (%)
	(% tel quel)	(% d.m.)	
Pumpkin naked seed oil cake			
OLINKA	11.2	12.1	7.93
SB	11.1	12.1	8.28
F1 OLINKAxG	10.3	11.2	8.31
F1 OLINKA x 371B	14.1	15.2	7.63
GLEISDORFER EXPRESS	14.7	16.2	8.84
GLEISDORFER DIAMANT	13.5	14.8	8.77
K2	12.4	13.5	8.15
Pumpkin husk seed oil cake			
OLIVIJA	12.7	13.8	8.06
DAKI 802	10.0	11.0	8.80
K1	13.3	14.2	8.23

Data are the means ± standard deviation values (n = 3)

As shown in Table 1, the moisture contents in the cakes were rather steady, ranging from 7.63 to 8.84%. On the other hand, the results regarding the amounts of oil remaining in the cakes were not so homogenous – the contents varied from 11.0 to 16.2 % d.m. and were considerably high. Berenji (2007) reports that the content of remained oil may vary from 10 to 18%, depending on the pressing method.

Table 2 shows the composition and content of fatty acids in the samples of oil extracted from press cakes. As for the composition of fatty acids, pumpkin seed oil belongs to the oleic-linoleic type. As seen in Table 2, oleic acid content ranged from 37.1±0.11 to 43.9±0.04%, while that of linoleic acid, belonging to ω-6 fatty acids, varied from 30.8±0.09 to 44.5±0.015%. In addition, the presence of a small percentage (0.1 do 0.3%) of α-Linolenic acid, an ω-3 fatty acid, was detected, which increased oil's nutritional value. The measured data are in accordance with the results obtained for the oil extracted from seeds, reported by various authors (Schuster, 1983; Wentzel, 1987; Karlović et al., 2001; Fruhwirth et al., 2003; Vukša et al., 2003; Vujasinović et al., 2010). Namely, the literature does not provide specific data on the composition of fatty acids in the oil extracted from pumpkin seed press cake.

Considering that the composition of fatty acids is a major indicator of both nutritional value and oxidative stability of vegetable oils, it was important to determine the total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) content as well. A higher SFA content contributes to oxidative stability, but from the nutritional point of view and in terms of preventing coronary disease, oils with higher MUFA and PUFA contents are preferred. The total content of unsaturated fatty acids in the examined oils was on average four times higher than that of saturated fatty acids.

Table 2. Fatty acid composition (% w/w) of the oil extracted from pumpkin seed press cake

Fatty acid	***Oli extracted from naked pumpkin seed cake***						***Oli extracted from husk pumpkin seed cake***			
	Olinka	SB	F1 Olinka x G	F1 Olinka x 371B	Gleisdorfer Express	Gleisdorfer Diamant	K2	Olivija	DAKI 802	K1
Myristic acid C14:0	nd	0.1±0.03	0.2±0.04	nd	0.2±0.03	0.2±0.00	nd	nd	nd	nd
Palmitic acid C16:0	12.9±0.09	11.6±0.06	11.8±0.09	11.4±0.02	11.5±0.11	15.3±0.30	13.3±0.09	11.9±0.12	15.5±0.10	11.2±0.02
Stearic acid C18:0	6.2±0.05	5.1±0.01	6.2±0.03	6.1±0.09	6.2±0.09	9.3±0.02	5.6±0.06	6.5±0.10	5.3±0.08	5.2±0.08
Oleic acid C18:1	43.9±0.04	42.9±0.02	40.7±0.06	41.7±0.01	37.5±0.08	43.5±0.03	43.6±0.11	42.3±0.05	37.1±0.109	39.2±0.10
Linoleic acid C18:2	36.7±0.06	40.2±0.20	40.8±0.07	40.3±0.31	44.3±0.04	30.8±0.09	37.3±0.01	39.0±0.12	41.7±0.17	44.5±0.15
Linolenic acid C18:3	0.1±0.12	0.1±0.21	0.2±0.19	0.3±0.21	0.2±0.29	0.1±0.10	0.3±0.19	0.2±0.18	0.1±0.16	0.2±0.22
Behenic acid C22:0	nd	nd	nd	0.4±0.17	0.1±0.00	0.5±0.15	nd	nd	0.3±0.00	nd
SFA	19.1±0.14	16.8±0.10	18.2±0.16	17.9±0.28	18.0±0.23	25.5±0.59	18.9±0.15	18.5±0.22	21.1±0.18	16.4±0.10
MUFA	43.9±0.78	42.9±0.67	40.7±0.66	41.9±0.78	37.5±0.55	43.7±0.71	43.6±0.69	42.3±0.70	37.1±0.70	39.2±0.69
PUFA	36.8±0.79	40.3±0.88	41.0±0.79	40.6±0.66	44.5±0.59	30.9±0.60	37.6±0.88	39.2±0.69	41.8±0.70	44.7±0.78

Data are the means ± standard deviation values (n = 3)

Total tocopherols were measured and the results are shown in Figure 1.

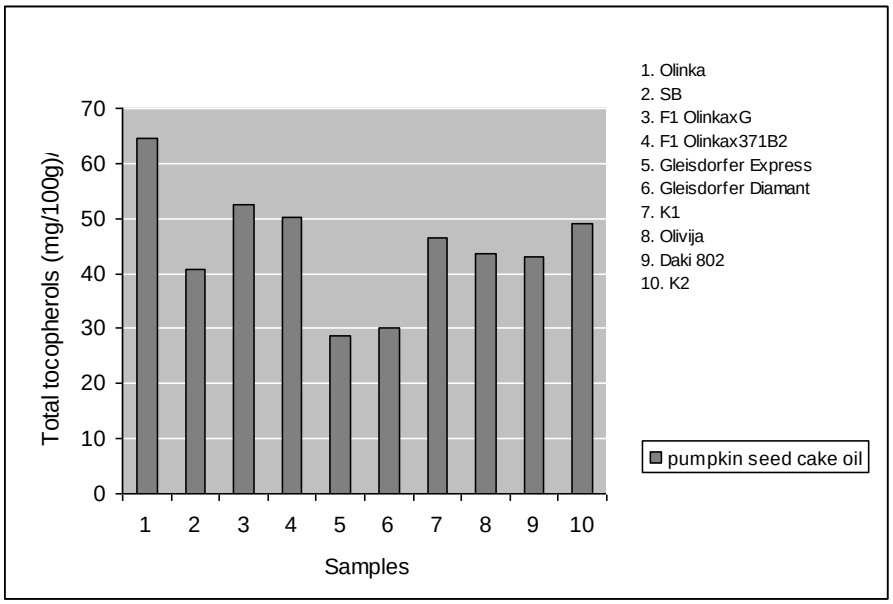

Figure 1. Total tocopherols content in oil from pumpkin seed press cake

The total content of tocopherols in the oil extracted from pumpkin seed press cakes was relatively high, ranging from 28.76 to 64.53 mg/100g and contributing to the nutritional value of the samples. The content was highest in the oil obtained from the Serbian “Olinka” pumpkin cake, and lowest in the oil extracted from the cake remained after pressing the seeds of the Austrian “Geisdorfer Express” hybrid. γ-Tocopherol as the dominant tocopherol isomer in all samples, accounting for 73.64 – 85.28% of total content.

CONCLUSIONS

From the point of view of the composition of fatty acids all samples regardless of the type of cake used for their extraction corresponded to the oleic-linoleic type, which indicates a high nutritional value of the oil. Considering that the amount of tocopherol detected in the examined oil was considerably high, we can draw a conclusion that a large percentage of tocopherol remains in the cake. Therefore, the oil left over in the cake should be considered for use, taking into account its nutritional value and its high presence in the cake.

ACKNOWLEDGEMENT

The authors would like to thank the Ministry of Education and Science, Republic of Serbia, for the grant 46010.

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RHEOLOGICAL PROPERTIES OF FUNCTIONAL SPREAD OF HULL-LESS PUMPKIN SEED FLOUR AND WHEAT FIBRE GEL

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ABSTRACT: Rheological properties of the spread based on hull-less pumpkin seed flour were determined. Sunflower oil, as fat phase of control sample, was totally replaced with colloidal wheat fibre gel. The gels were prepared in different concentrations, 0.1 to 10%, and were added in different amounts, 60, 70 and 80%, calculated on the total mass of the spread. Wheat fibres formed liquid gels in water at low concentrations, 0.1 and 0.5%. Stable gel structures were obtained at concentrations above 1% and had anti-thixotropic flow properties. The spreads of hull-less pumpkin seed flour and wheat fibre gel were thixotropic systems. Statistically insignificant ($p>0.05$) changes pointed that application of fat replacer, compared to control spread, regardless amount and concentration of the gel, did not affect the yield stress of the spreads. The interaction of these factors, as well as their separate influence, were statistically significant ($p<0.05$) for rheological parameters thixotropic loop area and tan δ. Creep and recovery compliance increased with increasing amount of the gel in spread composition. Optimal rheological properties of the spreads were achieved with low concentrated gel, 0.5 or 1%, in amount of 70%.
Key words: *wheat fibre gel, hull–less pumpkin seed flour, functional spread, rheological properties*

INTRODUCTION

Hull-less oil pumpkin, *Curcubita pepo L.*, is the form of ordinary pumpkin with specific hull-less seeds. Hull-less pumpkin seed is rich in oil (35-50%), proteins (30-40%), minerals and vitamins. The hull-less pumpkin oil is an important source of linoleic acid (40-57%), plant sterols, carotenoids, phenols and vitamin E. (Kreft et al.,2002) Mechanical process is usually used to produce the pumpkin oil. The hull–less pumpkin seed is cold pressed by hydraulic presses or screws. During that mechanical process significant amount of by-product, the oil cake is obtained. The oil cake is rich in proteins (60-65%), with a number of essential amino acids. (Čorbo et al., 2007; Tarek et al., 2001). Also, the proteins are specific by strong pharmacological activity antifungal, antibacterial, antioxidant and anti-inflammatory. (Peričin et al., 2008). The hull-less pumpkin seed cake, unlike the majority of oilseed cakes, does not contain antinutritive ingredients such as chlorogenic acid from sunflower seeds or tannins and phytic acid from rape seed.

Milled pumpkin oil cake represents flour with valuable nutritional composition and good sustainability. The flour contains metabolically very adoptable proteins, minerals (P, K, Mg, Mn, and Ca) and antioxidants. The content of all amino acids, except lysine, in the hull-less pumpkin seed flour is higher than in soy flour, and it is in accordance to suggested daily amount and quality of protein for human consumption by the World Health Organization. (Radočaj, 2011)

The hull-less pumpkin seeds flour can be especially used in food products that are in accordance with the principles of functional food. Functional food products contain biologically active micro and macro nutritients (vitamins, minerals, fibres), that provide adequate nutrition and health effects. The development of functional food is directed to a variety of low-energy products. Also, the trend of reducing the fat content in food products led to the development of fat replacers.

Fat replacers should imitate all functional characteristics of the fat, sensory properties (taste and odour), rheological properties (viscosity, consistency and texture), thermostability and

emulsifying properties that provide liposoluability of flavour and vitamins. The energy value of fat replacer should be significantly lower than energy value of the fat, 0 kcal/g is preferably. (Akoh, 1998)
The chemical structure of fat replacers can be similar to the chemical structure of proteins, fats or carbohydrates. Carbohydrate based fat replacer include native starch, modified starches, maltodextrin, polydextrose, gums and dietary fibres. (Cho and Prosky, 1999). Important technological characteristics of dietary fibre that can act as fat replacers are water holding capacity, fat binding capacity, adequate viscosity, gel formation capacity, chelating capacity and influence on the texture of the product. Soluble fibres form a gel structure by networking of polymer units. The water or other solvent is trapped into gel structure. Properties of the gel and gel formation ability depend on many factors: concentration, temperature, presence of certain ions and pH. Some fibres have a synergistic effect during the formation of a gel and are usually used in conjunction with starch, xanthan gum or carrageen. (Borderias et al., 2005). Dietary fibres can be used as fat replacer at numerous food products, baked goods, confectionery, cereal products, meat and dairy products, spreads, sauces, salad dressings, frozen products and that depend on its chemical composition. (Jones, 1996)
The aim of this work was to determine the influence of wheat fibre based fat replacer on the rheological properties, flow characteristics and viscoelastic properties, of the spread with hull-less pumpkin seed flour. The spreads contained colloidal wheat fibre gel instead the fat phase. The gels were prepared in different concentrations from 0.1 to 10% and were added into the spreads in different amounts 60, 70 and 80%, calculated on the total mass of the spreads. The possibility to obtain a nutritionally and biologically valuable food product that belongs to the group of functional low-energy products is examined.

MATERIAL AND METHODS

The hull-less pumpkin seed flour was obtained by oil producer “Linum" doo, Čonoplja, Serbia. The flour contained oil (15.41%), protein (55.02%), moisture (7.16%), cellulose (3.49%), ash (8.28%) and carbohydrates (10.63%). The fractions of the flour that passed through sieve with diameter 1 mm were used.
Fat replacer based on wheat fibres was used. It is commercial product named Vitacel® WFG HS73 by J. Rettenmaier & Sonho GMBH + CO Rosenberg Germany, and it is consisted of colloid wheat fibres (70%) with maltodextrin (30%). Applied emulsifier was sunflower lecithin, Topcithin® SF produced by Cargill, Italy. Salt (NaCl) and distilled water were used also.

Preparation of wheat fibre gels

Different concentration (0.1%, 0.5%, 1%, 5% and 10%) of wheat fibres were dispersed in distilled water and were homogenized 4 min at homogenizer (Ultraturaks T-25, Janke Kunkel) with S25 N-18G tool at 6500 min^{-1}. The solutions were stored at 25°C for 24 hours to form a gel.

Preparation of the spreads

The control spreads contained hull-less pumpkin seed flour, sunflower oil, salt (NaCl) and emulsifier-sunflower lecithin. The spread with 40% of oil, including the oil content of the flour, calculated on the total spread mass was optimal from rheological and textural aspect by previous analysis. Fat phase of the test spreads was totally replaced with wheat fibre gel. The amounts of gel were different, 60, 70 and 80% calculated on the total mass of the spread in the aim to achieve adequate consistency of the spread. The amounts of salt and the emulsifier were 0.5% and 0.4%, respectively, calculated on the total spread mass.

Rheological measurements

Rheological properties of the gels and spreads were determined by rotational viscometer HAAKE Rheostress RS600 (Thermo Electron Corporation, Karlsruhe, Germany) with plate–plate sensor PP60 Ti (plate diameter was 60 mm and gap 1 mm). The measurements were

done at 25 ± 0.1°C. Flow properties were defined by hysteresis loop method. The samples were exposed for 3 min to shear rate ramped up from 0-100 s^{-1}, following 3 min the shear rate was constant at 100 s^{-1} and finally ramped down to 0 s^{-1} for 3 min. The flow curves were characterised by yield stress τ_0 and thixotropic loop area A_0. Storage modulus (G') and loss modulus (G") were defined by dynamic oscillatory measurements in the range of linear viscoelastic regime (LVE). The moduli were observed during increased frequency from 1 to 10 Hz and at constant shear stress of 5 Pa and expressed as value tan δ = G"/ G'.
Viscoelastic response of the samples at constant stress, as well as their behaviour after removing the stress, were determined by creep and recovery test. The test was performed in the LVE regime in which the deformation amplitude was proportional to applied stress amplitude. The creep time with constant stress of 5 Pa was 150 s and the recovery period after removing the stress was 450 s. Statistical analysis of the data and significant differences at the significant level 0,05 for n=3, were determined by the ANOVA procedure and Duncan's multiple range tests.

RESULTS AND DISCUSSION

Rheological properties of gel

Low concentrations of wheat fibre, 0.1% and 0.5% formed lightly gelled systems in water. Stable gel structures were obtained with higher concentrations of fibres, 1%, 5% and 10% (Figure 1). Table 1 represents rheological parameters of the gels.

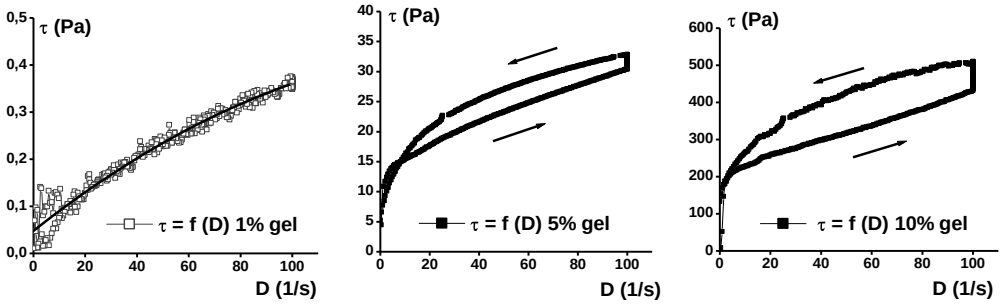

Figure 1. Flow curves of gels with 1, 5 and 10% of wheat fibre

Table 1. Rheological parameters of the wheat fibre gels

Gel concentration [%]	τ_0 [Pa]	A_0 [Pa/s]	tan δ
1	–	–	0.567
5	4.551	-290.13	0.232
10	5.191	-5456.67	0.205

The 1% gel showed the typical pseudoplastic flow properties. The concentrations of 5 and 10% of wheat fibre formed anti–thixotropic gels that occurred as result of internal structure established by linkages due to elongation and parallel placement of fibre filaments in shear gradient. The parameters of dynamic oscillatory measurements, tan δ, for high concentrations of the gels (5 and 10%) were lower than for 1% gel. Thus, the contribution of elastic components increased at these viscoelastic systems with high concentration of wheat fibre.

Rheological properties of the spreads

Figure 2 represents flow curves of the spreads with different amount of wheat fiber gel. The applied gels varied in concentration (0.1, 0.5, 1, 5 and 10%).

The obtained spreads with wheat fibre gel as well as the spread with sunflower oil were thixotropic systems. Statistically insignificant ($p>0.05$) changes pointed that application of the fat replacer, compared to the control spread, regardless amount and concentration of the gel did not affect yield stress of the spreads and for all spreads it was approximatelly 5 Pa.
Rheological measurements for spreads with 10% gel in amounts of 60 and 70% could not be performed because of high viscosity of these spreads. The flow ability had only the spread with 80% of this gel. The rheological parameter of the spreads, thixotropic loop area, was influenced by amount and concentration of the gel. Statistical analysis ($p<0.05$) confirmed the interaction of these factors, as well as their separate influence.

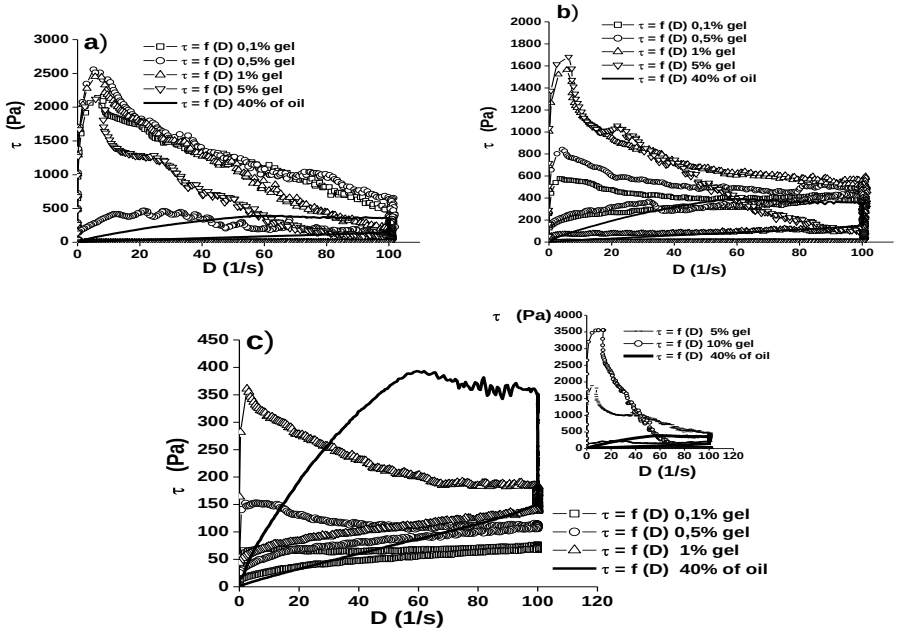

Figure 2. Flow curves of the spreads with a) 60%, b) 70% and c) 80% of wheat fibre gel

Figure 3a represents the changes of thixotropic loops area of the spreads affected by gel concentration at constant amount of the gel. The structure of the spreads with wheat fibre gel compared to the spread with sunflower oil was more complex, stronger and with more order. The influence of gel concentration on thixotropic loops area was the most evident at spreads with high amount of the gel, 80%. Increase in gel concentration contributed to firmness and the consistency of the spreads, thus the thixotropic loops area increased. The flour solids at the spreads with enough gel were coated and embedded in network gel structure. Decrease in amount of the gel reduced the influence of the gel concentration. With small amounts of the gel, 60%, high values of thixotropic loops area were caused by friction between solids and significant viscosity of the systems.

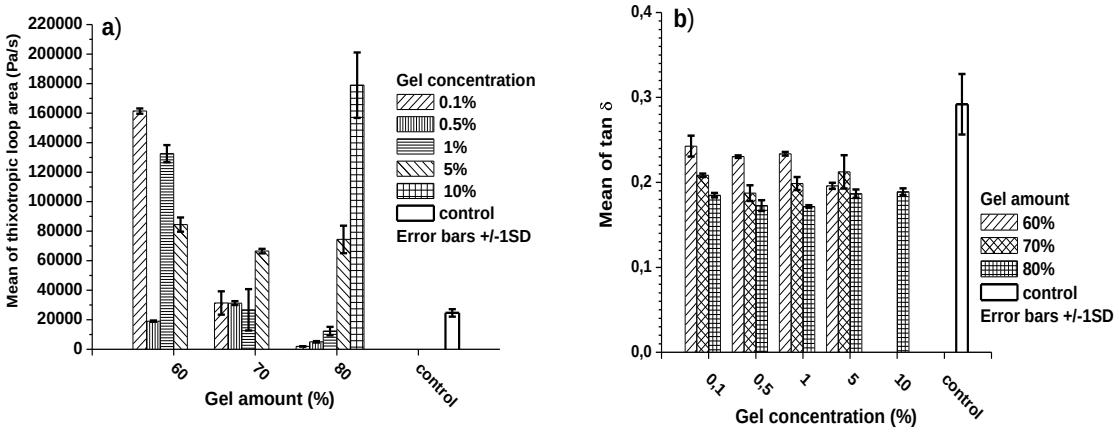

Figure 3. a) The influence of gel concentration on thixotopic loop area; b) the influence of gel amount on tan δ

The contribution of elastic and viscous components to viscoelastic properties of the spreads was determined by dynamic oscillatory measurements. Loss modulus G'' versus storage modulus G' represents value tan δ (Figure 3b). The influence of gel concentration on value tan δ was statistically insignificant ($p>0.05$), but the influence of gel amount and the influence of interaction of these factors were statistically significant ($p<0.05$). The increase in amount of the gel decreased the value tan δ, thus the difference between modulus decreased. All the spreads were viscoelastic systems (tan δ<1) with dominant storage modulus G' and similar behaviour caused by less or more firm structure.

The consistency of the spreads was defined by creep and recovery analysis. Viscoelastic response to applied stress described the compliance of the examined spreads. Figure 4 represent the influence of two factors, the amount of the wheat fibre gel and the concentration of applied gel, on the creep and recovery curves of the spreads. Increase in amount of the gel at all gel concentrations decreased the consistency of the spreads. More gel caused higher compliance and softer consistency of the spreads that were difficult to recover. The gel acted as continuous phase that coated solid particles and reduced their interactions. The spreads with same gel amount with increase in gel concentration were less susceptible to applied stress, accordingly increase in gel concentration led to increase in firmness of the spreads.

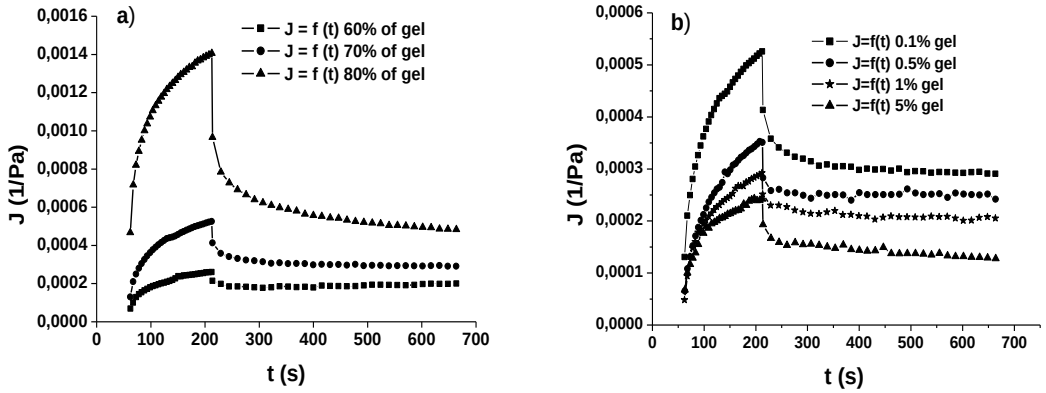

Figure 4. The influence of a) the amount of the wheat fibre gel; b) the concentration of applied gel on the compliance of the spreads

CONCLUSION

Colloidal wheat fibers gel can replace the fat phase of the examined spreads. Obtained spreads were viscoelastic systems that exhibited thixotropic flow properties and certain recover ability. The concentration of applied gel and amount of the gel defined the structure and consistency of the spreads. Optimal rheological properties of the spreads were achieved with low concentrated gel, 0.5 or 1%, in amount of 70%. A low-energy functional spread can be obtained.

ACKNOWLEDGEMENTS

This research was done within Project entitled "Cookies and creckers with functional characteristics aimed for consumers with special dietary needs" financed by the Provincial Secretariat for Science and Technological Development.

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ANTIOXIDANT POTENTIAL OF BASIL (*Ocimum* spp.) GENOTYPES GROWN IN SERBIA

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ABSTRACT: Basil (*Ocimum spp.* L., family *Lamiaceae*) has been grown traditionally in Serbia for decorative, medicinal, and as a spice used in cooking. In Orthodoxy it is used in religious services , and in India and Pakistan basil it is an herb dedicated to the Goddess Tulsa. Secondary metabolites from *Ocimum* species possess an exceptional biological activity and have: bactericide, fungicide, repellent, antiinflammatory, antioxidative, antidiarrheic, chemopreventive and radioprotective effects. The antioxidant potential of different genotypes was examined of Basil from Serbia according to their anti-radical capacity (Free Scavenging Capacity - RSC).The analyzed genotypes included: *Genovese, Lattuga, Fino Verde, Holanđanin, Compact, Lime*, *Cinnamon, Siam Queen, Blu Spice, Purple Ruffles, Purple Opal, Osmin* and *Holy Red.* The DPPH assay was used to measure stable free radicals. The analysis of these genotypes indicate that *Purple Opal* has the highest antioxidant potential of IC_{50}=0,14 mg/ml.
Key words: *basil genotype, antioxidant potential, DPPH, Serbia*

INTRODUCTION

Basil (*Ocimum spp.* L., family *Lamiaceae*) has been grown traditionally in Serbia as a decorative, medicinal, seasoning and ritual herb. Basil was introduced to Serbia by monks, who received the plant during a pilgrimage in the 12th century. Since then it has been used in Orthodox Church ceremonies. Additionally, the Serbian nation has grown basil in home gardens for centuries. This reputable 'holy' plant has been used as a decorative, repellent, in traditional medicine, and in culinary arts (Jelačić et al., 2010).

The genus *Ocimum* (*Lamiaceae*) consists of 30-150 species, distributed in the tropical regions of Asia, Africa and Central and South America. This highly variable species has a complex taxonomy which is a consequence of the inter-specific hybridization and polyploidy of the species (Grayer et al., 1996). Their morphological diversity has been developed through centuries of cultivation, and their delineation has been further complicated by the existence of chemotypes within the species that do not differ significantly in morphology (Simon et al., 1990; Paton et al., 1999).

Secondary metabolites from *Ocimum* species possess an exceptional biological activity and have: bactericide, fungicide, repellent, antiinflammatory, antioxidative, antidiarrheic, chemopreventive and radioprotective effects (Opalchenova et al., 2003; Pascual-Villalobos and Ballesta-Acosta, 2003; Lukmanul Hakkim et al., 2008; Gajula et al., 2009; Runyoro et al., 2010).

Basil herb (*Basilici herba*) is used in traditional homeopathic medicine for treating a large number of diseases. It is used as a carminative, sedative, lactagogue, for increasing appetite, for cough reduction and in cosmetology as an exceptional antiseptic. In the perfume industry the essential oil of basil (*Basilici aetheroleum*) is used, as well as some of its components, for the production of perfumes and other products (Simon et al., 1990).

Basil is cultivated in Serbia mostly in home gardens. In most cases the morphology of the varieties grown in Serbia are large leaved and small leaved, green in color, with linalool being the dominant component of their essential oil (Jelačić et al., 2010). Through many

years of systematic collection a large number of genotypes has been compiled which have not been cultivated in Serbia until now. This process has introduced many new varieties and has enlarged the number of Basil accessions in our gene bank.

MATERIAL AND METHODS

Plant material and cultivation conditions

Seeds of 13. *Ocimum spp.* cultivars were planted in the greenhouse in March of 2010, in polystyrene containers with 40 holes, and stuffed with commercial substrate (Beatović et al., 2006). Seedling production lasted 48 days. During May of 2010, nursery plants were transplanted into the experimental field in a randomized complete block design with four replicates in a 50x50 cm model, at Surčin, Serbia (44° 49' N, 20°17' S, altitude 96 m, degraded chernozem). Plants were harvested at the full flowering stage in July of 2010. Plants were air dried in the shade, and then packed and stored.

Seeds of *O. basilicum* cultivars (Table 1.) were obtained from the Institute for Crop Sciences of the Faculty of Agriculture in Belgrade and the Plant Gene Bank of Serbia where plants were designated and deposited under DB codes (Beatović et al., 2008)

Table 1. Introduced genotypes of basil

Genotypes	Code	Taxonomic classification of the genus *Ocimum*
Genovese	DB001	*O. basilicum* subs. *basilicum* var. *basilicum*
Lattuga	DB002	*O. basilicum* subs. *basilicum* var. *difforme*
Fino Verde	DB003	*O. basilicum* subs. *basilicum* var. *basilicum*
Holanđanin	DB004	*O. basilicum* subs. *basilicum* var. *basilicum*
Compact	DB005	*O. basilicum* subs. *basilicum* var. *minimum*
Cinnamon	DB006	*O. basilicum* subs. *basilicum* var. *glabratum*
Lime	DB007	*O.* x *cirtiodorum* (*O. basilicum* x *O. americanum*)
Siam Queen	DB008	*O. basilicum* subs. *basilicum* var. *citriodorum*
Blu Spice	DB009	*O. basilicum* (*O. americanum* x *O. basilicicum* var. *purpurescens*)
Purple Opal	DB010	*O. basilicum* subs. *basilicum* var *purpurescens*
Purple Ruffles	DB011	*O. basilicum* subs. *basilicum* var *purpurescens*
Osmin	DB012	*O. basilicum* subs. *basilicum* var *purpurescens*
Holy Red	DB013	*O. sanctum* (syn. *O. tenuiflorum*)

Radical scavenging activity

Air-dried aerial parts of 13 basil genotypes were extracted with MeOH for 48 h at room temperature. The ratio between plant material and solvent was 1:20, w/v. These extracts were measured using the DPPH assay.

The DPPH method was originally published by Molyneux (2004). The method is simple and rapid for determining the antioxidative activity of food, plant extracts and chemical compounds by using DPPH as the stable radical. The reaction mixture (1ml) contained 500µl of daily prepared DPPH solution in methanol (150µM) and 500µl of various concentrations (1, 0.5, 0.25, 0.125, and 0.0625 mg/ml) of the tested methanol extract of basil. After thorough mixing, the solutions were kept in the dark for 20 min at room temperature. Thereafter, the absorbance was measured at 517 nm. All tests were performed in triplicate, with trolox and ascorbic acid as a positive control. The percent inhibition of the DPPH radical by basil extracts was calculated according to formula:

$$\% \text{ Inhibition} = [(A_{blank} - A_{test}) / A_{blank}] \times 100$$

The IC_{50} values were calculated by linear regression of plots where the abscissa represented the concentration of tested samples and ordinate the average percent of inhibition activity from three separate tests. Data are presented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Basil is used as a spice in food preparation, and since it is an important ingredient it is necessary to identify its antioxidative potential. This study tested the antioxidant potential of

13 basil genotypes which differ according to their morphological characteristics and composition of essential oil (Beatović et al., 2010).
In Table 2. is presented the DPPH radical scavenging activity of the tested Basil genotypes.

Table 2. DPPH radical-scavenging activity of tested Basil genotypes

No.	Genotypes	DPPH radical scavenging activity IC_{50} (mg/ml)
1	Genovese	0.68 ±0.01
2	Lattuga	0.33 ±0.02
3	Fino Verde	0.32 ±0.02
4	Holanđanin	0.31 ±0.01
5	Compact	0.31 ±0.01
6	Cinnamon	1.82 ±0.04
7	Lime	0.19 ±0.01
8	Siam Queen	0.21 ±0.01
9	Blu Spice	0.33 ±0.01
10	Purple Opal	0.14 ±0.01
11	Purple Ruffles	0.28 ±0.01
12	Osmin	0.18 ±0.01
13	Holy Red	0.25 ±0.02
14	ASA	0.003 ±0.00
15	TROLOX	0.004 ±0.00

The Purple Opal genotype had the greatest antioxidant activity 0.14 mg/ml among the 13 genotypes tested. This genotype belongs to the group anthocyanin colored Basils. The remaining anthocyanin colored genotypes also showed high antioxidant activity including; Osmin, Purple Ruffles and Holy Red (Table 2.).
Of the genotypes that belong to the green colored group (those without anthocyanin coloring) the Lime genotype had the greatest antioxidant activity 0.19 mg/ml. The large leaved genotype Genovese had a lower antioxidant activity 0.68 mg/ml, while the lowest activity was from the Cinnamon genotype 1.82 mg/ml (Figure 1.).

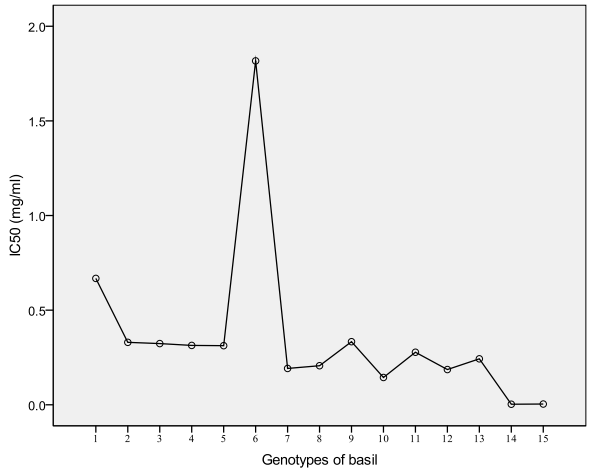

Figure 1. Basil genotype IC_{50} values

Many authors have analyzed the antioxidant capacity of basil (Lee et al., 2005; Hussain et al., 2008; Kwee and Niemeyer, 2011).
Most studies have concentrated on analyzing essential oils, whereas less have focused on the directly analyzing the herbal material. Hinneburg et al. (2006) studied the antioxidant potential of Basil grown in Finland. When comparing their result of 0.49 mg/ml it can be concluded that Basil grown in Serbia has a higher antioxidant capacity.
While Lukmanul Hakkim et al. (2008) had a greater antioxidant capacity from *Ocimum gratissimum* grown in India compared to the genotypes analyzed in this study.

Figure 2 and 3. Herbal material of tested Basil genotypes

CONCLUSIONS

Basil is widely used in the culinary arts and the food processing industry. It is used as an aroma to improve the taste, smell, and digestibility of food. Basil leaves are used often as a spice therefore it is important to know its antioxidative potential and associated health benefits. The Purple Opal genotype had the greatest antioxidative capacity of all 13 tested genotypes, while Osmin and Lime genotypes were also considered to have a high antioxidative capacity. These results show that a significant antioxidative potential can be achieved by cultivating introduced Basil genotypes under the agroecological conditions in Serbia.

ACKNOWLEDGEMENTS

This paper is a result of the research within the projects III 46001 “Razvoj i primena novih i tradicionalnih tehnologija u proizvodnji konkurentnih prehrambenih proizvoda sa dodatom vrednošću za domaće i svetsko tržište (Development and implementation of new traditional technologies in production of competitive food products with the value added for domestic and world market) and OI 173015 (Biotechnology in vitro – crop, medicinal and endangered plant species). The realization of the project was financed by the Ministry of Education and Science Republic of Serbia.

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COMPARISON OF LOW MOLECULAR WEIGHT GLUTENIN AND GLIADIN SUBUNITS IN SELECTED WHEAT VARIETIES

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ABSTRACT: The most important role in wheat protein complex belongs to gluten with its components: glutenin and gliadin. Majority of conducted and published research related to investigation of glutenin and gliadin fractions composition is directed to high molecular weight subunits and their relation to expressed technological properties of wheat varieties. Differentiation of wheat varieties in relation to low molecular weight subunits composition with molecular weight of up to 30 kDa gains in importance under the fact that some of low molecular protein fractions found in wheat express bioactive properties with confirmed curative and preventive properties proven *in vivo* or/and *in vitro* in relation to cardiovascular diseases, cancer, immune and other disorders. Determination of glutenins and gliadins of wheat varieties from different regions was conducted by LoaC (Lab-on-a-chip capillary electrophoresis) method with previous separation of glutenin and gliadin fractions. PCA was utilized to identify the varieties which are characterized with the most expressed differentiation in the low molecular weight glutenins and gliadins composition. Ten wheat varieties, including wide spread in production but also those with high differentiation of composition and shares of protein fractions in the interval from 13 to 28 kDa were compared, and the significance of differences was analyzed with ANOVA test.
Key words: *wheat, functional properties, bioactive peptides, electrophoresis*

INTRODUCTION

Today wheat is one of the most important crops in the world, considering the area of approximately 240 million of hectares under cultivation and the total yields (Mastilović et al., 2011). These facts make wheat also one of the most suitable potential sources of functional ingredients, including bioactive proteins and peptides, both from the technological and economical points of view (Belović et al., 2011). From the technological point of view, wheat proteins can be divided to proteins that are constituents of gluten and those that are not. Gluten proteins (gliadins and glutenins) are the most abundant and major proteins (approximately 85% of total proteins) (Korhonen and Pihlanto, 1998) located in the endosperm of wheat, and they are considered to play a dominant technological role. Low and middle molecular weight glutenin subunits (LMW-GS) constitute about one third of total kernel protein content, and approximately 60% of total glutenins, while other part of kernel is constituted of less abundant high molecular weight glutenin subunits (HMW-GS) (Song and Zheng, 2007). Among wheat proteins with low molecular weights there are proteins that can potentially have bioactive properties (Korhonen and Pihlanto, 2003).
The fact that bioactive peptides can help reduce global epidemic of chronic diseases that is responsible for 58 millions of premature deaths per year (Wang et al., 2007) should not be neglected. Many researches carried out by biological *in vitro* and/or *in vivo* tests are dedicated to consideration of action of bioactive peptides encrypted in protein sequence/protein hydrolysates from plant sources. They have physiological effect on the major body systems − the cardiovascular, digestive, endocrine, immune and nervous system. The most investigated peptide of plant origin with anticarcinogenic action is lunasin, which is firstly discovered is soybean, and later in some cereals and pseudocereals, such as wheat, barley, rye, rice and amaranth (Belović et al., 2011).

Immunomodulatory peptides, beside improvement of immune system function, can enhance mucosal immunity in the gastrointestinal tract and reduce allergic reactions (Hartman and Meisel, 2007). In addition to commonly investigated anticarcinogenic action of lunasin, Dia et al. (2009) reported its anti-inflammatory action.
The aim of research presented in this paper was to identify wheat varieties that are distinguished by structure and proportion of glutenins and gliadins in range of molecular weights from 13 to 28 kDa, with emphasis on identification of varieties which could based on low molecular weight glutenins and gliadins content and structure be potential source of peptides with bioactive properties.

MATERIAL AND METHODS

Wide range of wheat varieties was investigated, including current varieties present in the assortment and genetic material from different parts of the world, on the basis of glutenin and gliadin fractionation and their separation using chip electrophoresis technique.Total number of analyzed samples was 247 wheat varieties. First phase of investigation of samples was their preparation, actually grinding and fractionation of anatomical parts of the grain during milling. Wheat was milled using Sedimat mill (Brabender Ohg., Germany). Reagents used for fractionation and denaturation of wheat flour proteins were: SDS (10%), 4 x STACKING GEL BUFFER (0.5 M tris (base) – Cl pH 6.8) and 2 x TREATMENT BUFFER (0,125 M tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol).
Fractionation of wheat flour proteins was carried out on the basis of their different solubility. First step of fractionation was the extraction of albumins and globulins, considering their solubility in water and saline solution, respectively. Further procedure included extraction of:

- Gliadins (prolamins) – proteins soluble in 70% ethanol;
- Glutenins (glutelins) – proteins soluble in dilute solutions of acids and alkalis.

Separation of proteins in previously prepared samples was performed using chip electrophoresis technique on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with Protein 230 Plus Lab-on-a-Chip kit. For each investigated wheat variety, analysis was conducted in two independent replications. Preparation of samples and their deposition on chips was performed according to protocol for analysis with Protein 230 Plus Lab-on-a-Chip kit. Results of chip electrophoresis were expressed by software in two different ways – as quantitative profiles and simulated gel images. Qualitative analysis included the determination of molecular weights of separated protein fractions, and quantitative analysis included determination of concentrations of each defined protein fractions. All samples were analyzed in three replications. Results of chip electrophoresis were presented graphically (electrophoregrams) and in table (values) for each sample of wheat flour.
Software used for processing of experimental values obtained for low molecular weight protein proportions (13-28 kDa) of analyzed wheat varieties was Microsoft Office Excel 2007 (Microsoft Corporation, 2007) and STATISTICA (StatSoft, Inc. (2011), version 10.0 (www.statsoft.com)). Data bases obtained by STATISTICA were processed using PCA (Principal Component Analysis) multivariate technique and ANOVA (analysis of variance), followed by Duncan's multiple range test in order to compare means of selected samples at 5% significance level.

RESULTS AND DISCUSSION

Characteristics of data base created from analysis results for low molecular weight glutenin and gliadin fractions in all 247 wheat varieties are presented in Table 1.
In observed interval of molecular weights from 13 to 28 kDa, on the basis of analysis of large number of different wheat varieties, three characteristic intervals with low molecular weight glutenin and gliadin peaks can be noticed:

- Interval from 13 to 19 kDa with highest average content of glutenins and gliadins and highest concentration of varieties in range from 15 to 17 kDa.

- Interval from 19 to 24 kDa with highest average content of glutenins and gliadins and highest concentration of varieties in range from 20 to 22 kDa.
- Interval from 24 to 28 kDa with highest average content of glutenins and gliadins and highest concentration of varieties in range from 25 to 27 kDa.

Table 1. Characterization of data base on low molecular weight glutenins and gliadins in wheat varieties

Interval of molecular weight, kDa		Average content, ppm	Min, ppm	Max, ppm	Standard deviation	Coefficient of variation
13-28	**Glutenins**	2147.15	316.66	7705.8	1030.18	48.0
	Gliadins	7094.61	0	22866.49	3188.84	44.9
13-19	**Glutenins**	1179.34	0	3887.27	658.18	55.8
	Gliadins	30.44	0	2744.76	276.2	907.3
19-24	**Glutenins**	584.72	0	2959.93	398.29	68.1
	Gliadins	139.45	0	3252.15	451.75	323.9
24-28	**Glutenins**	383.09	0	3845.62	625.45	163.2
	Gliadins	628.62	0	9348.37	1450.13	230.6

Processing of complex data from obtained data base included analysis of glutenin and gliadin fractions by emphasized intervals of molecular weights in ranges from 13 to 19, 19 to 24 and 24 to 28 kDa by principal component analysis. Results of principal conmponent analysis for glutenins and glidins are presented in figures 1 and 2 respectively.
For both, glutenins and gliadins the first two principal conponens accounted to over 85 % variability of the whole population. Based on the separation of varieties in the factor plane 5 varieties which were the most distiguishing among others were selected for gliadins and for glutenins and illustrative and comparative display and analysis of glutenins and gliadins electrophoregrams for selected varieties was provided.

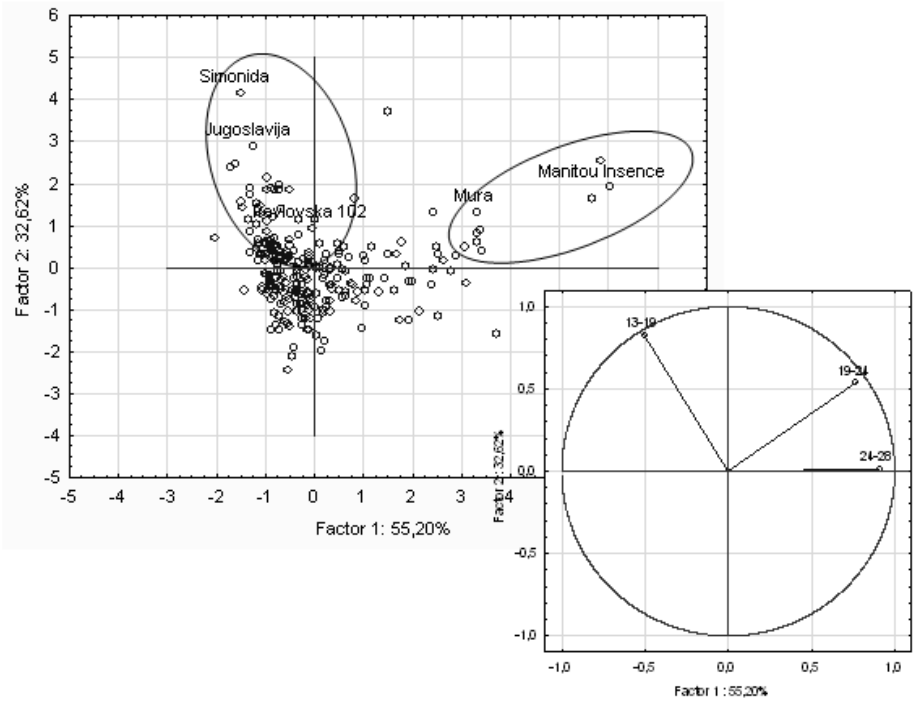

Figure 1. Principal component analysis of obtained data base for content of low molecular weight glutenins in intervals 13-19, 19-24 and 24-28 kDa

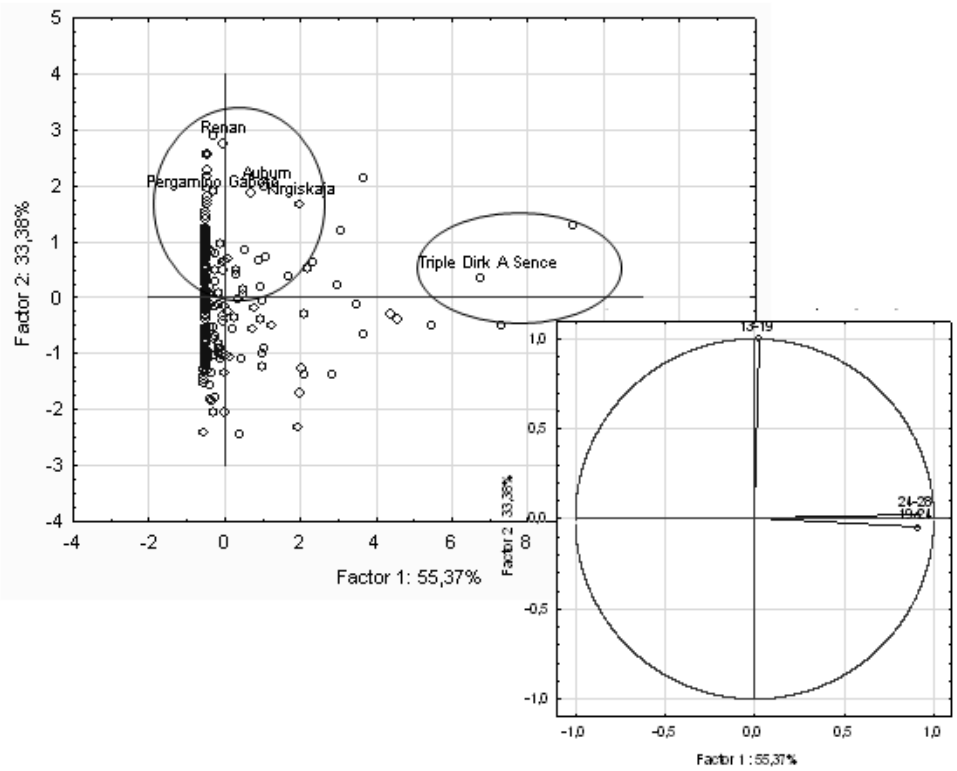

Figure 2. Principal component analysis of obtained data base for content of low molecular weight gliadins in intervals 13-19, 19-24 and 24-28 kDa

Five varieties for each type of wheat proteins were selected on the basis of principal component analysis. Their basic electrophoregramic data were analyzed and presented.

- For glutenins, varieties Jugoslavija, Simonida, Manitou Insence, Pavlovska 102, Mura;
- For gliadins, varieties Auburn, Renan, Pergamino Gaboto, Kirgiskaja, Triple Dirk A Sence.

Concenrning glutenins varieties Simonida, Jugoslavija and Pavlovska 102 were distinguished in interval of molecular weights from 13 to 19 kDa, variety Manitou Insence in both intervals from 19 to 24 kDa and 24 to 28 kDa and variety Mura in interval from 19 to 24 kDa.
Concerning glutenins variety Triple Dirk A Sence was distinguished in the intervals from 19 to 24 and 24 to 28 kDa. Varieties Pergamino Gaboto, Kirgiskaja, Auburn and Renan were distinguished in the interval from 13 to 19 kDa.
Content of low molecular weight glutenins and gliadins of varieties selected by distinguished intervals of molecular weights is presented in Tables 2 and 3, respectively.

Table 2. Low molecular weight glutenins content of selected varieties by identified molecular weight intervals

Variety	13-19 kDa		19-24 kDa		24-28 kDa	
	ppm	Number of peaks	ppm	Number of peaks	ppm	Number of peaks
Jugoslavija	3558.39$^{(a)}$	3	906.06$^{(c)}$	1	154.34$^{(c)}$	1
Simonida	3887.27$^{(a)}$	2	1059.32$^{(c)}$	1	0$^{(d)}$	0
Pavlovska 102	1514.53$^{(b)}$	1	835.00$^{(d)}$	1	225.65$^{(c)}$	1
Mura	926.50$^{(c)}$	3	2077.48$^{(b)}$	2	1420.58$^{(b)}$	1
Manitou Insence	800.24$^{(c)}$	2	2959.93$^{(a)}$	1	3845.62$^{(a)}$	1

(a), (b), (c), (d), (e) *Values with the same superscript within a column belong to the same homogenous group.*

Table 3.Low molecular weight gliadins content of selected varieties by identified molecular weight intervals

Variety	13-19 kDa		19-24 kDa		24-28 kDa	
	ppm	Number of peaks	ppm	Number of peaks	ppm	Number of peaks
Auburn	11313.72$^{(b)}$	2	0$^{(c)}$	0	3159.93$^{(c)}$	2
Renan	13495.89$^{(a)}$	2	0$^{(c)}$	0	780.27$^{(d)}$	1
Pergamino Gaboto	11006.67$^{(b)}$	3	0$^{(c)}$	0	0$^{(e)}$	0
Kirgiskaja	10458.63$^{(b)}$	2	1016.06$^{(b)}$	1	4022.82$^{(b)}$	1
Triple Dirk A Sence	7528.83$^{(c)}$	1	2105.73$^{(a)}$	1	8094.13$^{(a)}$	1

(a), (b), (c), (d), (e) *Values with the same superscript within a column belong to the same homogenous group.*

Appearance of electrophoregrams of selected varieties is presented on Figure 3.
Separated glutenin fractions (Figure 3) in investigated interval of molecular weights from 13 to 28 kDa confirm that the flour sample of variety Simonida was distinguished by interval of lower molecular weight, while Manitou Insence and Mura were distinguished by interval of higher molecular weights, with significant quantitative differences in mentioned range with regard to other varieties.
Separated gliadin fractions (Figure 3) in investigated interval of molecular weights from 13 to 28 kDa, confirm that the flour samples of varieties Kirgiskaja, Auburn and Renan were distinguished by interval of lower, while variety Triple Dirk A Sence was distinguished by interval of higher molecular weights. Variety Pergamino Gaboto had very characteristic appearance of electrophoregram, which was different from other investigated varieties.

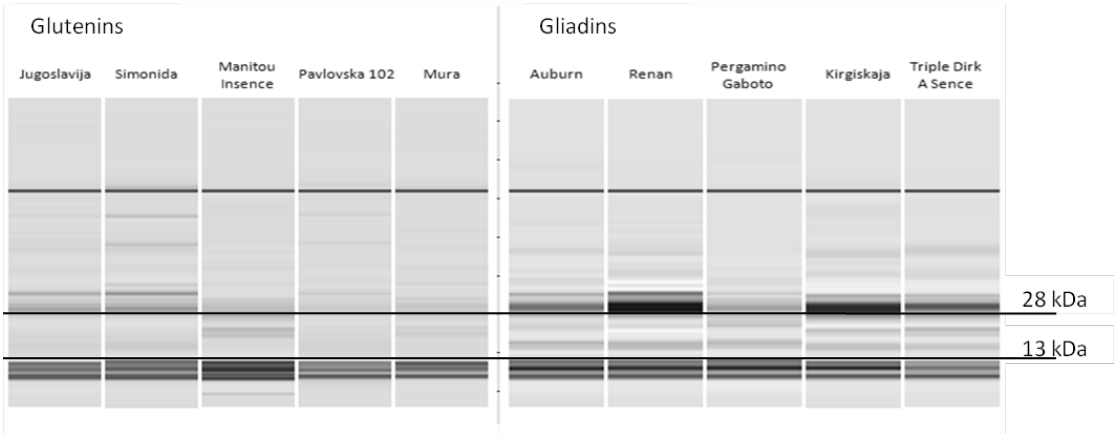

Figure 3. Appearance of electrophoregrams of selected wheat varieties

CONCLUSIONS

Wheat as a carrier of a number of biologically active compounds with preventive activity occupies a significant place as a carrier of functional ingredients in terms of frequency of consumption of bread and pastries in the diet. Cultivars originating from various parts of the world show differences in structure and proportion of both glutenin and gliadin fractions in the range of molecular weights for that literature data indicate the existence of fractions with bioactive properties. Generally, the range of lower molecular weights (13-28 kDa) in the structure of the wheat proteins, amounts of glutenin fractions are less that amounts of gliadin fractions.

ACKNOWLEDGEMENTS

This paper is a result of the researches within the Project No. III 46001 financed by the The Ministry of Education and Science, Republic of Serbia.

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EFFECTS OF PESTICIDES IN HONEY AND OTHER BEE PRODUCTS ON HUMAN HEALTH AND ENVIRONMENTAL SUSTAINABILITY

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ABSTRACT: Honey is regarded as a natural and safe product for human that free of any chemical contaminants. However, today some of honey is produced in an polluted environment by different sources of contamitants. These are present in air, water, soil and plants and are transported to beehives and bee products by bees. Pesticide is a general name of chemicals which consist of insecticides, acaricides, fungicides and herbicides. Nectar and pollen collected from pesticide-treated flowers can result in contaminated honey. The residues of pesticides detected in bee products are mostly organochlorines, organophosphates, carbamates and neonicotinoids. Plant-derived pesticides, called botanicals, have also been developing quickly. Consumers in Europe are concerned about pesticide residues in food. The small amounts of pesticide residues remain in the honey and other products constituting a potential risk for the human health. Pesticide use also raises a number of environmental concerns.
Key words: *pesticises, honey, pollen, honey bee, residue*

INTRODUCTION

We will be needed more food to feed world population predicted to be 9 billion by 2050. The development of public and private food safety standards has been driven by the numerous scandals like dioxin that occured during the last years (Willems et al., 2005). In recent years public concern about the safety of foods of animal origin has heightened due to residue problems originated from contaminants, dioxins and pesticide resistance. Residues are substances that can ocur in foods after using veterinary drugs and pesticides in beekeeping and in agriculture. Pesticide residues in foods are unwanted phytosanitary products or derivates. Pesticides are designed to control pests and disease-causing organisms. They are chemicals that control insects, weeds, fungi and other pests that destroy almost half of the world's food crops. However, pesticides are poisonous. Therefore they must be applied correctly to protect bees in pollination (Fraser, 2004). Many pesticides contain persistent organic pollutants, which persist in the environment and accumulate in the fatty tissue of animals, including humans. The accumulation of these chemicals in the body can pose a serious health risk to living organisms, and have been implicated in causing cancer, disease, hormonal imbalances and reproduction problems, including birth defects (Griffin, 2011). They can be also present in non fatty products, such as water, fruits and vegetables. Organochlorine pesticides (OCPs) can be present in honey by the means of treatment of some plants. Pesticides might be introduced into honey from nectar or pollen collected by honeybees from contaminated blossoms. They and their metabolites can accumulate in wax of the comb (Fernandez-Muino et al., 1995). However, for every one dolar that is sepent on pesticides for crops yields approximately four dollars in crop saved. Pesticides also reduce biodiversity, destroy habitat and cause pesticide resistance.
Pollination is an essential part of the ecology and bees are the most important pollinators (Martin, 2003). Honeybees are beneficial to human by producing honey, wax and pollen, besided pollination (Kolonkaya et al., 2002). They are biological indicators, picking up chemicals and other pollutions from their environment both external and internal to their hives (Wu et al., 2011). Bee products have the image of being natural, healthy and clean. Asia is the greatest honey producer. Total honey production in the world is approximately 1.3 million tones. However, today honey and other bee products such as pollen, wax and

propolis are produced in an environment polluted by different sources of contaminants (Chlebo, 2006). The contamination sources can be environmental and apicultural. Residue in honey (Piro and Mutinelli, 2003). There is a need to improve preventitive methods to reduce or avoid accumulation of contaminants in the apiary infrastructure in aim to produce clean honey and apiary products (Barel et al., 2011). Good Agricultural Practice (GAP) and Integrated Pesticide Management (IPM) can reduce risk to human, bees and the environment (McKee, 2003). The aim of this paper is to give information about pesticides, their action and effects on bees and bee products, human and the environment. It also aims to give preventition methods and legislations for pesticide residues in bee products.

PESTICIDES AND THEIR CHEMICAL STRUCTURE

Pesticides are used in agriculture and farming practices to eradicate insects, rodents, weeds and other forms of pests(Anonymous, 2012a). They can be divided to two classes, broad-spectrum and narrow-spectrum pesticides. Broad spectrum pesticides kill many kinds of pests, while narrow-spectrum pesticides do just the opposite. Narrow-spectrum pesticides are developed to kill specific organism types. However, systemic pesticides work differently. They penetrate to the inside of a plant travelling along its absorbtion path. These poisons work by poisoning the pollen and nectar of flowers and this can kill needed pollinators (Anonymous, 2012b). Pesticides include insecticides, fungicides, herbicides, acaricides, bactericides and nematodices. The residues of pesticides detected in bee products are mostly insecticides, acaricides, fungicides and herbicides. The total amount of pesticides applied worldwide is currently put at 2.5 million tonnes. Of this 50-60% is herbicides, 20-30% insecticides and 10-20% fungicides (Anonymous, 2012a). Pesticides used on various crops are divided to groups based on their chemical structure (Bogdanov et al., 2003; Anonymous, 2012a):

- Organochlorines pesticides (OCPs): They are hazardous because of their bioaccumulative action into the food chain, to remain stable for years and to move the environment. DDT, lindane, hexachlorocyclohexane, aldrin, dieldrin, endrin, heptachlor and endosulfan are in this group.
- Organophosphorus pesticides (OPPs): This specific class of pesticides is of relatively high toxic for humans. This includes dialiphos, trichlorophon, dichlorvos, parathion, malathion, ronnel, methoate and several others. These chemicals break down quickly when exposed to weather and very hazardous for mammals than the OCPs.
- Carbamates: They don't leave residue on foods. Their mode of action is similar to OCPs but their insecticidal activity is more selective.
- Pyrethroids: These chemicals are similar to pyrethrum but do not break down quickly as the natural products. These substances were proved toxic to bees but the concentrations of residues detected in honey were very low.

Beekeepers are frequently compelled to use varroacides to avaoid colony death from *Varroa* mites and residue (Johnson et al., 2010). The Varroacides can be divided into three categories: synthetic organic pesticides (pyrethroid, fluvalinate, amitraz and fenpyroximate), natural product pesticides (thymol, menthol and other herbal essential oils) and organic acid pesticides (formic acid and oxalic acid).

PESTICIDE RESIDUES IN HONEY AND OTHER BEE PRODUCTS

Effects of pesticides on honey bees

Honey bees make an important contribution to sustainable agriculture and the environment as the main pollinators (Anonymous, 2012c). Pesticides are responsible for disappearing of honey bees in recent years. Pesticides are stored in honey bee's body while they are harvesting nectar and pollen from flowers (Griffin, 2011; Kolankaya et al., 2002). Most of the time, accaricide treatments for *Varroa* lead to residues in hives (Chauzat and Faucon, 2007). The recent sequencing of the honey bee genome provides a possible explanation for the

sensitivity of bees to pesticides. The honey bee genome is markedly deficient, including cytochrome P450 monooxygenases, glutathione-s-transferaes, and carboxylesterases (Claudiones et al., 2006). Pesticide residues as ppb and occasionally ppm can be detected in hive matrices when bees forage in any conventional agricultural or urban settings (Johnson et al., 2010). Delayed development was observed in bees reared in containing high levels of pesticides (Wu et al., 2011).

Pesticide residues in bee products

Systemic pesticides are usually incorporated into the soil or onto seeds and more up into the stem, leaves, nectar, and pollen of plants. A systemic pesticide may incorporate into fruits, pollen and nectar of the treated plants (Anonymous, 2012d). However, bee products from natural vegetation contain lesser residues than agricultural fields. Most of the acaricides such as coumaphos, amitraz and fluvalinate leave residues in hives. In honey, residue levels are low, but accumulation of several pesticides in wax could lead to synergistic toxic effects on bees, also, the persistence of acaricides in wax favor the appearence of acaricide resistent mites (Barel et al., 2011). The medicines used in varroa treatment leave residue in honey, wax and propolis. Fluvalinate and flumethrin, fat soluble compounds, hazard with wax. They become the active ingredient and we do not get rid of it until the wax is burned. If these compounds are used several years in succession than the residues build up and some moment they become so large that there are traces to be found in the honey. Already after one year, there are such large traces in the wax. After one or two years the compounds can be found in honey. The compouns alternate to these chemicals (formic acid and lactic acid) are to be found naturally in honey. With the recommended dosages they do not produce a larger content in honey (Jorgensen, 2005). The relatively low concentrations of pesticides in honey seem to be due to a filtering effect of bees (Chlebo, 2006). Pesticides can baund strongly with propolis (Jorgensen, 2005). Bogdanov (2006) reported the main contamination risks for the different bee products. Antibiotics in honey and Royal jelly, persistent lipophylic acaricides in wax, persisitent lipophylic acaricides and lead in propolis and pesticides in pollen are the risk factors.

Results of experiments were conducted to determine the pesticide residues in bee products:

In Turkey, residues of OCPs and OPPs insecticides were detected in honey and pollen but not in propolis. The levels were below the level of toxicity (Kolankaya et al., 2002). In a Spain and Portugal study, 50 honey samples were analysed to determine 42 residues of pesticides. Most of the pesticides found in honey were OCPs (Blasco et al., 2003). In a Swiss study, 27 honey samples, produced between 1998 and 2001 were analysed. The results of this research showed that pesticides originating from agriculture and the environment are not a significant contamination source for honey and beewax (Bogdanov et al., 2003). In Switzerland, 27 samples of pesticide in wax have been analysed. All the samples contained at least one pesticide residue. The content was three to four times higher than that from the same year for fluvalinate, and for brompylate was twice as much, with contents of 3.7 mg and 1.3 mg per kg, respectively (Jorgensen, 2005). In a Danish study, 35 honey samples were tested for the present of 37 different pesticides including flumethrin. No measurable traces of pesticides were to found at all. However, traces of pesticide residue were found in fifteen of the samples of thirty two wax samples were collected from Danish market (Jorgensen, 2005). In Slovenia, monitoring of 20 honey samples for the presence of 14 insecticides showed no detectable residues of insecticides (Chlebo, 2006). A multiresidue analysis of 16 insecticides and acaricides and two fungicides showed that 14 of the searched for coumaphos and endosulfan residues were the most frequently (61.9, 52.2 and 23.4% of samples, respectively). Coumaphos was found in the highest average quantities (792,6 microgram^{-1}). Residues of cypermethrin, lindane and deltamethrin were found in 21.9, 4.3 and 2.4% of samples, respectively (Chauzat and Faucon, 2007). Martel et al. (2007) found varroacide in wax, pollen and bee bread include amitraz, bromopropylate, coumaphos, flumethrin and fluvalinate. In France, a 3-yr field survey was carried out from 2002 to 2005. Residues of imidacplorid and 6-chlorinicotinic acid were the most frequently detected in pollen, honey, and honey bee matrices (Chauzat et al., 2008). Amitraz residue was also

found in various levels in six out of 32 honey samples in Turkey (Çobanoğlu and Tüze, 2008). Choudhary and Sharma (2008) found DDT and its isomers in honey samples produced in India. Residues of OPPs were not detected. Johnson et al. (2010) analysed one hundred twenty one different pesticides and metabolites. Pesticides were found in 887 wax, pollen, bees and associated hives.

EFFECTS OF PESTICEIDES ON HUMAN HEALTH AND THE ENVIRONMENT

In the EU no plant protection substances can be used unless if they have been scientificly established that they have no harmful effects on consumers and farmers. However, the WHO estimates that each year, 3 million farmers in agriculture in the developing countries experience severe poisoning from pesticides. Pesticide residues may cause harmful health effects including skin irritations, delayed or altered development, lung problems, nervous system disorders, liver-kidney damage, reproductive damage, endocrine and immune damage, and cancer (Anonymous, 2012e). The dangers of pesticides can start as early as fetal stage of life and cause growth problem (Anonymous, 2012b). The health effects of pesticides depend on the type of pesticide. OPPs and carbamates affect the nervous system. Others may irritate the skin or eyes. Some pesticides are carciogens. Others may affect the hormone or endocrine system in the body (Anonymous, 2012f). OCPs hydrocarbons (e.g. DDT) operate by disrupting the sodium/potasium balance of the nevre fibre. OPPs and carbamates operate through inhibiting the enzyme acetylcholinesterase, causing a variety of symptoms such as weakness or paralysis (Anonymous, 2012g). OCPs pesticides transfer from blossom to honey and finally to the consumer. They have been restricted or banned in agriculture because of their persistence and bioaccumulation in the environment. However, these type pesticides are stil found in soil from which they continue to cycle through the environment. Compounds that are soluble in water are removed from the blood stream by the kidneys and excreted in the urine. Because DDT and its relatives are fat-soluble, they are not excreted in the urine. Modern pesticides are much less persistent than DDT and are not prone to bro-accumulation (Johnson and Jadon, 2010). The OCPs are banned in developed countries. However, they are stil widely used in developing countries because they are relatively cheap (Anonymous,2012a). More than 95% of insecticides and herbicides can reach non-target species, air, water and soil. They can be carried to other areas by wind. Pesticides also reduces biodiversity, damages habitat and decrease pollinators.

METHODS TO DECREASE PESTICICE RESIDUE IN BEE PRODUCTS

The beekeepers are the first subjects in the production chain to avoid honey contamination. They are mainly entered in the quality of bee product, but they needed more effective control tools (Piro and Mutinelli, 2003). Pesticide residue problem is on second of consumer perception list of possible risk. Food additives are on top of this list. Alll pesticides must be adequately tested, evaluated and authorised for potentionally adverse effects on foods, human health and the environment before they are licensed. Some pesticides are restricted. However, the restricted and not authorised pesticides carry a greater level of risk (Fraser, 2004). In the EU 0.01 mg/kg pesticide in honey is limit. To avoid pesticide residues should be used outside the bloom period or at least, not during the foraging time of bees. Beekeepers can also avoid residue by placing their hives more than 3 km from agricultural plants treated with pesticides (Chlebo, 2006). Farmers should do this application by using IPM. it is the best available pest control system. Farmers and beekeepers should also apply Push Pull strategy which uses a mixture of behaviour-modifying stimuli to manipulate the distribution and abundance of insects. The overuse, misuse and overuse of peticides must be controlled. Farmers should use cultivation practices including polyculture and crop rotation to manage the pest damage. Farmers must prefer biological pest control methods including natural predators or parasites of the pests. Beekeepers and farmers must be in good accordance during pesticide application period. It is beter to use new products have been produced in

recent years as alternative to hazardous pesticides. New pesticides include biological and botanical derivates and alternatives that are though to reduce health and environmental riks.

LEGISLATIONS FOR PESTICIDE RESIDUES IN BEE PRODUCTS

In Europe, recent EU legislation has been approved banning the use of carciogenic, mutagenic or toxic to reproduction. The EU approves the use of pesticides and sets tolerence levels. MRLs for pesticide residues permitted in or on a food are often 10 to 1,000 times less than the amount needed to pose a health risk. The amount of pesticides on fruits, vegetables and crops can be decreased by washing, peeling, blanching and cooking but people consume honey directly (Fraser, 2004). The substances for any type of animal or food that must be monitored were divided into two groups in EU legislations. The categories are shown in the table have to be monitored in bee products (Piro and Mutinelli, 2003):

- GROUP A- Substances having anabolic effect and outhori**z**ed substances:
  (6) Compounds included in Annex IV to Council Regulation (EEC) 2337/90
- GROUP B. Veterinary drugs and contaminants
  (1) Antibacterial substances icluding sulphanomides, quinolenes
  (2) Other veterinary drugs
  (c) Carbamates and pyrethroides
  (3) Other substances and environmental contaminants
  (a) Organochlorine compounds including PCBs, (b) Organophosphorus compounds (c) Chemical elements

However, in Europe, accaricide residues in wax are not regulated (Chauzat and Faucon, 2007). In Turkey, the same categories of substances also have to be monitored in bees and bee products. Turkey has been conducting an EU Leonardo da Vinci Transfer of Innovation project titled 'Beekeeping European Environmental Sustainability BEES' with Holland, Belgium, Italy, Poland and Hungary to produce learning materials for beekeepers for all about sustainable beekeeping (http//:www.beeseurope.eu). In 2008, the EU issued new and revised MRLs for the roughly 1,100 pesticides ever used in the world. The revision was intended to simplfy the previous system, under which certain pesticide residues were regulated by the Commission, other were regulated by the Member States, and others were not regulated at all (Anonymous, 2008). The new Regulation covers pesticides currently or formerly used in agriculture in or outside the EU. For crops grown outside the EU, MRLs are set on request of the exporting country. Developing country exports must meet importer's MRLs or perhaps in future the international recommendations under WTO rules.

In conclusion, the main contamination for bee products come from apicultural practices and agriculture. To avoid harmful effect of pesticides on environment, beekeepers, bee products, and human educational programs and projects should be developed by the public health organisations. Especially, developing countries need to assistance in establishing pesticide residue standards. Consumers in these countries must be also educated about health concerns associated with pesticides. It is also necessary for developed countries that importing bee products from third countries.

ACKNOWLEDGEMENTS

We thank EU LLP LdV ToI 'BEES' Project (2010-1-TR1-LEO05-16698) and 'Center for European Union Education and Youth Programmes of Turkey' to support this paper.

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DOMESTIC BALKAN DONKEYS' MILK: MICROBIOLOGICAL, CHEMICAL AND SENSORY PROPERTIES

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ABSTRACT: The aim of this study was to investigate microbiological, chemical and sensory properties of raw donkeys' milk from an autochthonous breed originated from Serbia. Changes in microbial flora during the six days of storage at 4 ºC were monitored by enumeration of total count of bacteria, yeasts and moulds, coagulase positive staphylococci, lactic acid bacteria, bacterial endospores, *E.coli*, *C. perfringens* and *Salmonella* spp. Descriptive method was used for determination of sensory properties. Descriptive terms were developed to describe appearance (colour), flavour, taste and aftertaste. Chemical analyses included determination of protein, milk fat, dry matter, lactose and vitamin C content. After five days of storage at 4 ºC microbiological quality of the raw Domestic Balkan donkeys' milk was in accordance with the requirements of European Regulation. The obtained results indicate strong antimicrobial activity of the tested milk and longer shelf-life in comparison to other raw milk sources. Sensory properties of the tested milk were in accordance with changes in microbial flora. High vitamin C content, low protein and milk fat content make the tested milk a valuable potential component of functional food.
Key words: *donkeys' milk, microbiological properties, sensory properties, chemical composition*

INTRODUCTION

The therapeutic effects of donkeys' milk have been known since ancient times, while nowadays the research is addressed towards its nutrient composition and health effects. Donkeys' milk has lower content of fat, protein and inorganic salts, and higher content of lactose in comparison to bovine milk. Low casein content, high percentage of essential amino acids, protein and lipid profiles similar to those of human milk (Fantuz et al., 2001; Salimei et al., 2004; Vincenzetti et al., 2008) favour donkeys' milk as a potential new dietetic food and a good alternative for infant nutrition in the case of cow's milk protein allergy (CMPA) (Iacono et al., 1992; Monti et al., 2007; Vita et al., 2007). Several studies reported potential anti-inflammatory effect (Ellinger et al., 2002; Jirillo et al., 2010) and positive effect of donkeys' milk on the prevention of atherosclerosis (Tafaro et al., 2007). Anti-proliferative and anti-tumor effects of some active compounds in donkeys' milk on human lung cancer cells have also been reported (Mao et al., 2009). High concentration of polyunsaturated fatty acids, vitamins A, B, C and low cholesterol level contribute to donkeys' milk functionality (Solaroli et al., 1993).

The Domestic Balkan donkey is an autochthonous breed primarily farmed in the Northern and Eastern regions of Serbia (Food and Agriculture Organization–Domestic Animal Diversity Information System, 2009; Kugler et al., 2008). The aim of this study was to investigate the chemical and sensory properties of Domestic Balkan donkeys' milk, as well as changes in microbial flora during the six days of storage at 4 ºC.

MATERIALS AND METHODS

Sample collection

Donkeys' milk samples were collected immediately after morning milking from "Zasavica" Special Nature Reserve. The milk obtained from eight manually milked donkeys was collected, mixed and kept in an ice box at 4 ºC during the transport. The milk sample was divided into three equal parts designated to study its microbiological, chemical and sensory properties. The milk sample assigned to microbiological examination was further divided into seven sub-samples of 50 mL each, intended to study the variability in microbial population during the six days of storage. One sub-sample was examined immediately, while the others were stored at 4 ºC and analysed on a daily basis. Donkeys' milk samples were collected three times following the same sampling procedure.

Determination of changes in microbial population during the storage

Changes in microbial flora during the six days of storage at 4 ºC were monitored by enumeration of total count of bacteria, yeasts and moulds, coagulase positive staphylococci, beta-glucuronidase-positive *Escherichia coli* and *Clostridium perfringens,* according to listed ISO methods: ISO 4833:2003, ISO 21527-1:2008, ISO 6888-1:1999/A1:2003, ISO 16649-2:2001, ISO 7937:2004, respectively. Enumeration of bacterial endospores was performed by incubation of previously heated (100 ºC, 5 min) milk samples on nutrient agar (Himedia, India) at 30 ºC for 72 h according to Serbian legislation (Sl. list SFRJ, no. 25, 1980). The lactic acid bacteria count was determined by incubation (30 ºC, 72 h) of inoculated Man, Rogosa and Sharpe (MRS) agar (LabM, United Kingdom). Coliform bacteria and *Enterobacteriaceae* were determined according to AOAC methods (AOAC Official method 996.02, 2002; AOAC Official Method 2003.01, 2003). Detection of *Salmonella* spp. was carried out according to the ISO 6579:2002/AC: 2006 method. All experiments were performed in triplicate.

Sensory analysis

Donkeys' milk samples were tempered at 15 ºC before analysis to allow the release of odors and flavors (Bandler et al., 1984). Approximately 25 mL of each sample was given to the assessors in 3-digits coded covered glasses. Sensory evaluation was performed during 6 days in order to test milk stability. Descriptive sensory analysis was performed by a trained eight-member panel. The panelists were selected according to SRPS ISO 8586-1(2002). Sensory evaluation was carried out in individual boxing in the laboratory according to SRPS ISO 8589 (1998).

Descriptive terms were developed for the assessment of sensory quality and intensity of colour, flavour, taste and aftertaste using terms from the lexicon of SRPS ISO 5492 (2000). The panelists used attributes, definitions and references from previous sensory studies of milk (Bandler et al., 1984). The following data scale was used: 1-typical, optimal quality; 2-neither good nor poor quality; 3-poor quality.

Chemical analysis

Protein, milk fat, lactose content and dry matter were determined by the IDF standard (Carić et al., 2000). The vitamin C content was determined by enzymatic method (Megazyme International Ireland 2011).

RESULTS AND DISCUSSION

The results of three independent experiments indicate that there was no significant difference ($P<0.05$) between total bacterial counts at the beginning (4.24 ± 0.90 – 4.79 ± 0.55) log cfu/mL and after five days of storage (4.59 ± 0.16–5.22 ± 0.76) log cfu/mL, whereas increase was observed on 6^{th} day (6.17 ± 0.40 – 6.57 ± 0.90) log cfu/mL. From the initial number of (2.08±0.03–2.10±0.90) log cfu/mL lactic acid bacteria count slightly increased to

(2.60 ± 0.31 – 2.95 ± 0.05) log cfu/mL after six days. Bacterial endospores remained at low level (<2 log cfu/mL) during the storage period. In the first experiment (milk collected in January 2011), *Enterobacteriaceae* and coliforms were not detected until the 2nd and 4th day, respectively, whereas in the milk samples collected in October and November 2011 these bacteria were not detected during all six days. The presence of *Salmonella* spp., *E. coli*, *C. perfringens*, coagulase positive staphylococci, yeasts and moulds were not observed in any of the analysed samples.

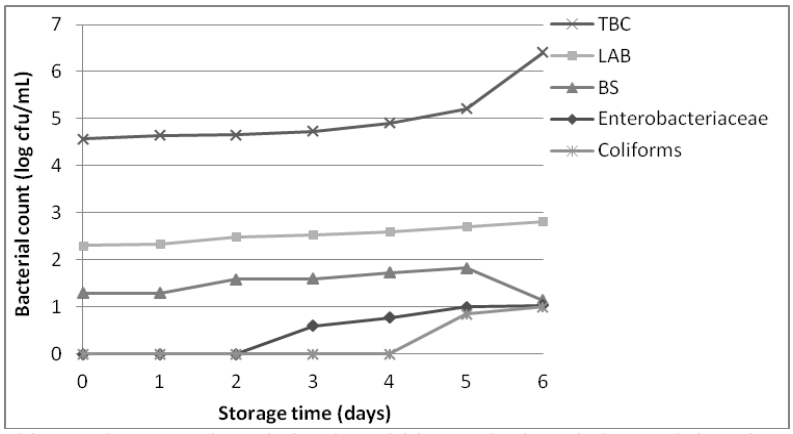

Fig. 1. Total bacteria count (TBC), lactic acid bacteria (LAB), bacterial endospores (BS) *Enterobacteriaceae* and coliforms counts in raw donkeys' milk during the six days of storage at 4 ºC (mean values of three independent experiments)

Typical milk colour did not change during all six days of assessment. Aroma was poorly expressed during the first three days, whereas on 4th day a milky-sweet flavour appeared and became more intense during the next two days leading to a lack of freshness. Panel members defined milk taste as a pleasant, with a slight sweetness originating from lactose. In terms of overall acceptability of taste, panellists did not observe any changes during the first three days of experiment. However, slightly bitter taste appeared on 4th day and intensified during the next days. For this reason, on 5th day milk was assessed as non specific according to its primary and residual bitter taste. Assessments of the sensory properties of the tested milk are shown in the Fig 2. The obtained results indicate that raw Domestic Balkan donkeys' milk is sensory acceptable during four days of storage at 4 ºC. This is in accordance with its microbiological quality.

Raw donkeys' milk has low protein and milk fat content and high lactose and vitamin C content (Table 2) which makes this milk optimal for new dietetic food formulation and human milk substituent in infant nutrition in the cases of bovine milk protein allergy.

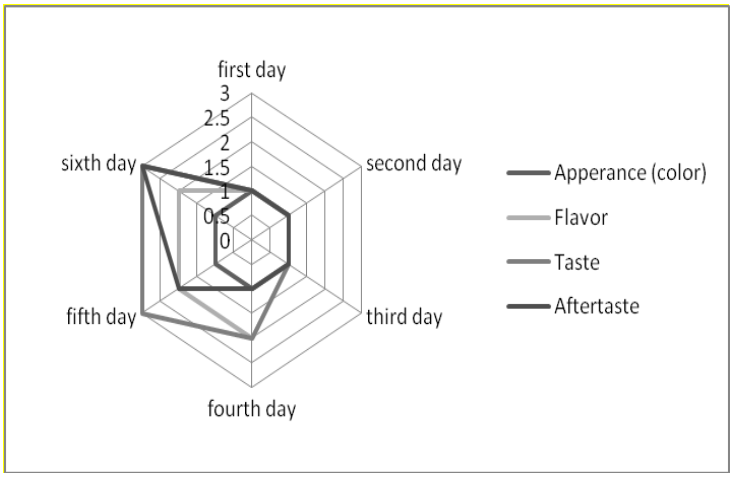

Fig. 2. Changes in the sensory properties of raw donkeys' milk during the six days of storage at 4 ºC

Table 2. Chemical composition of raw donkeys' milk

Chemical characteristics	(mean ± standard deviation)
Dry matter (%, w/w)	8.58±0.32
Protein content(%, w/w)	1.67±0.20
Milk fat content (%, w/w)	0.66±0.11
Lactose content (%, w/w)	7.07±0.14
Vitamin C (μg/mL)	32.95±1.38

CONCLUSIONS

Microbiological quality and changes in microflora during the six days of storage at 4 ºC indicate strong antimicrobial potential of raw Domestic Balkan donkeys' milk against the naturally occurring groups of microorganisms. After five days, microbiological quality of the tested milk was in accordance with the requirements of European Regulation, which indicates its longer shelf-life in comparison to other raw milk sources. Sensory properties of the tested raw milk were acceptable within four days of storage at 4 ºC. High vitamin C content, low protein and milk fat content make the tested milk a valuable potential component of functional food.

ACKNOWLEDGEMENTS

This work is a part of the National Project (TR–31029) financially supported by the Ministry of Education and Science, Republic of Serbia.

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ISOLATION AND BIOCHEMICAL PROPERTIES OF AEROMONAS HYDROPHILA HYDROPHILA IN RAINBOW TROUT

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ABSTRACT: *Aeromonas hydrophila spp.* is commonly present in water and in certain incidences they might cause diseases in fish and amphibians. Humans are infected by ingestion of these bacteria with contaminated food or water. *Aeromonas hydrophila spp.* might cause gastro-intestinal disorders and poisoning in humans. One of the major issues associated with *Aeromonas hydrophila* and incidence of gastro-intestinal diseases is accurate identification of aeromonades at the species level. Therefore, there is a need for a clear phenotypic scheme and biochemical characterization of the mentioned species.
Objective of this paper was to study the presence of *Aeromas hydrophila* in trout samples and to test biochemical characteristics of isolated strains.
For isolation of *Aeromonas hydrophila* species, 120 samples of rainbow trout, commercially available, were studied. *Aeromonas hydrophila* was isolated from fish muscle tissue and skin according to "Microbiological Methods for the Meat Industry", second edition (1991) Biochemical studies were performed on Aeromonas hydrophila medium, followed by oxidase test (+), catalase test (+), staining according to Gram (-) and vibriostat test agar (0/129, vibriostatic), as well as biochemical tests API 20E and API 20NE.
From the 120 studied rainbow trout samples, 12 strains of *Aeromonas hydrophila* were isolated, out of which seven strains (58.3%) of *Aeromonas hydrophila* belonged to group 1, whereas five strains (41,6%) belonged to *Aeromonas hydrophila* group 2. Out of 12 isolated strains, 11 (91.7%) had β-galactosidase. They were citrate and indol positive, sorbitol negative, arabinose positive, NO_2 -producing and reducing it to N_2. Then (83.3%) of 12 twelve isolated strains were Voges-Proskauer positive and melibiose negative, whereas eight (66.7%) were amigdalin and lysine-decarboxylase positive. It was established that seven of 12 twelve strains (58.3%) ferment glucose.
The obtained results demonstrate the need to supplement phenotypic schemes for identification of *Aeromonas hydrophila* strains originating from fish at the species level.
Key words: *Aeromonas hydrophila, Rainbow teout (Oncorhynchus mykiss), biochemical characterization*

INTRODUCTION

Representatives of genus *Aeromonas hydrophila* are facultative anaerobes, oxidase positive and gram- negative bacteria, whose main reservoir in nature is water (Anon., 2006). Hence, *Aeromonas hydrophila* species are often isolated from samples of fish crustaceans and shellfish, but also can be found in fresh food stuffs such as fresh meat, packaged products ready for consumption, cheese and milk (Karabasil et al., 1999; Palumbo, 1996; Panin, 1993; Buchanan and Palumbo, 1985; Tsai and Chen, 1996). As a consequence of alimentary poisoning caused by these bacteria, gastroenteritis occurs in healthy persons, but usually these are individual cases of disease without large epidemics. Symptoms of the disease are nausea, vomiting, stomach cramps and diarrhea (Ashdown and Koehler, 1993; Janda and Abbot, 1998).
Main factors of *Aeromonas hydrophila spp.* virulence are: secretion of exotoxins and endotoxins (lipopolysaccharide, LPS), presence of S-layer and fimbria or adhesins (Merino et al., 1996). According to results of Karabasil et al. (2002), studied filtrates of strains

Aeromonas hydrophila (9) and *A.sobria* (3), had cytotoxic effect on tissue culture of Vero cells, but the intensity of chages differed depending on the filtrate. Changes on Vero cells in sense of cytotoxic effect were caused by thermo sensitive toxin component.
According to literature data, in patients suffering from diarrhea, *Aeromanas* species were isolated in rate from 0.6 to 10% and predominantly in small children *(Janda and Abot,* 2010; Essers et al., 2000). According to epidemiological data from Spain, *Aeromonas hydrophila spp.* are in fourth place of all microbiological causes of gastro-intestinal diseases that have been notified each year during the period 1997 - 2006. (Anon., 2007.). This is indication of the importance of *Aeromonas hydrophila spp.* as cause of gastro-intestinal disorders and poisonings in humans, especially small children and immune compromised persons. In scientific community, there is no common position on factors causing the poisoning. Association between introduction of *Aeromonas hydrophila* through drinking water, food and diarrhea, still hasn't been identified (Anon., 2006).
Herra et al. (2006) point out geographical differences in distribution of *Aeromonas hydrophila* species and strains belonging to *Aeromonas hydrophila* (*Aeromonas hydrophila, A.bestiarum, A.salmonicida*) and *A.caviae* (*A.caviae, A.mediji, A.eucrenophila*) which can be found in sea and fresh water fish, as well as fresh water environment.
Due to its nutritional value and availability, trout from aquaculture in Serbia is very appreciated fish and represents valuable and generally accepted source of animal proteins in human nutrition (Trbović et al., 2011; Vranić et al., 2011). Some literature data indicate that fish meat in nutrition (20 g/day) reduces the risk of cardio-vascular diseases with fatal outcome by 7% (He et al., 2004). According the data from American Heart Association it was established that cardio-vascular patients should eat fish more than twice per week. Daily intake of 1 g EPA (eicosapentaenoic acid, pentane-eicosenoic acid C20:5 n-3) and DHA (docasahexaenoic acid, hexanedioci acid, C22:6 n-3) is necessary, (Lichtenstein et al., 2006) since long chain n-3 PUFA PNMK influence reduction of mentioned risk (Dewailly et al., 2007) as well as risk of auto-immune diseases (Zamaria, 2004), also malignant diseases (Terry et al., 2004) and diabetes (Nettleton and Katz, 2005).
Given the large presence of *Aeromonas hydrophila spp*. in nature, its psychotrophe nature and increasing significance as potential alimentary pathogen, objective of our study was to investigate the diffusion/prevalence of *Aeromonas hydrophila* in trout, as well as biochemical properties of isolated *Aeromonas hydrophila* strains.

MATERIAL AND METHODS

For the purpose of isolation of *Aeromonas hydrophila* 120 samples of Rainbow trout from retail stores were collected. Samples were put in sterile Stomaher bags and in chilled condition transported to the laboratory. Samples were processed within two to four hours from the moment of sampling in the Laboratory for biotechnological research and control of food safety and quality of the Institute of Meat Hygiene and Technology in Belgrade. *Aeromonas hydrophila* was isolated from samples of muscle tissue and skin of rainbow trout.
Isolation was carried out according to Microbiological Methods for the Meat Industry, 1991, of the Research Institute of the meat Industry from New Zealand. According to recommendations of previously mentioned edition, isolates were obtained in the following way: sample was transferred into 9 times the amount of APW, Alkaline peptone water and incubated for 24 h at 28 °C. Culture which grew in APW, was superficially placed on selective-differential foundation – SAA, Starch ampicillin agar, incubated for 24 h at 28 °C. After incubation, SAA was topped with Lugol solution preparation and colonies of yellow colour (amylase positive) with zone of illumination were further subcultured on Aeromonas hydrophila medium (AHM), incubated for 24 h at 28 °C, and subsequently oxidase test (+), catalase test (+), staining according to Gram (-) and vibriostat test agar (0/129, vibriostatic), as well as biochemical tests API 20E and API 20NE were carried out.

RESULTS AND DISCUSSION

In 120 studied samples of rainbow trout, 12 strains of *Aeromonas hydrophila* were isolated (A, I, J, B, C, K, L, D, M, E, F and G).
Collection of mobile *Aeromonas hydrophila*, consisting of 12 isolates, of which seven strains (58.3%) were *Aeromonas hydrophila* group 1, whereas five strains (41.6%) *Aeromonas hydrophila* were group 2. Strains A, B, C, D, E, F and were identified as *Aeromonas hydrophila* group 1, whereas strains I, J, K, L and M were identified as *Aeromonas hydrophila* group 2. Biochemical properties of isolated strains are presented in table 1, for *Aeromonas hydrophila* group 1 and in table 2, for *Aeromonas hydrophila* group 2.

Table 1. Biochemical properties of *Aeromons hydrophila* group 1.

Biochemical series		***Aeromonas hydrophila* group 1**						
		Isolated strain number:						
		A	**B**	**C**	**D**	**E**	**F**	**G**
1.	Presence of β galactosidase	+	+	+	+	+	+	+
2.	Presence of arginin hydrolase	+	+	+	+	+	+	+
3.	Presence of lysin decarboxylase*	-	+	+	-	+	-	-
4.	Presence of ornitin decarboxilase	-	-	-	-	-	-	-
5.	Citrate*	+	+	+	+	-	+	+
6.	Production of H_2S	-	-	-	-	-	-	-
7.	Urease	-	-	-	-	-	-	-
8.	Presence of tryptophan desaminase	-	-	-	-	-	-	-
9.	Indol	+	+	+	+	+	+	+
10.	VP*	+	-	-	+	+	+	+
11.	Dissolving gelatin	+	+	+	+	+	+	+
12.	Presence of citochrome oxidase	+	+	+	+	+	+	+
13.	Glucose fermentation*	-	+	-	-	+	+	-
14.	Manithol fermentation	+	+	+	+	+	+	+
15.	Inositol fermentation	-	-	-	-	-	-	-
16.	Sorbitol fermentation*	-	+	-	-	-	-	-
17.	Rhmanose fermentation	-	-	-	-	-	-	-
18.	Saccharose fermentation	+	+	+	+	+	+	+
19.	Melibiosis fermentation	-	-	-	-	-	-	-
20.	Amygdalin fermentation *	+	+	+	-	+	-	+
21.	Arabinose fermentacija *	+	+	+	+	+	+	-
22.	TSI	yellow	yellow	yellow	yellow	yellow	yellow	yellow
23.	Production of NO_2	+	+	+	+	+	+	+
24.	Reduction into gas N_2	-	-	-	-	-	-	-
25.	Mobility	+	+	+	+	+	+	+
26.	Growth on McConkey agar	+	+	+	+	+	+	+
27.	Hemolysis on blood agar*	+	+	+	-	+	+	+
28.	m-Aeromonas hydrophila medium	yellow	yellow	yellow	yellow	yellow	yellow	yellow
29.	OF/ F	+	+	+	+	+	+	+
30.	OF / O	-	-	-	-	-	-	-

*Note: * Non-specific reactions for group 1*

One of the major issues in associating *Aeromonas hydrophila* originating from fish with gastro-intestinal disorders and diseases is accurate identification of aeromonades/Aeromonas hydrophila at the level of species (Euzeby, 2010; Figeras et al., 2010; Minana-Galbis et al., 2010). Because of the lack of clear phenotype scheme, biochemical characterization of described species is relatively complicated. Presently, phenotype identification scheme by Abot et al., (2003) is used, who suggested use of Moeller-decarboxylase and dihydrolase reactions for identification and grouping of

Aeromonas hydrophila species. In this way, five groups with set of biochemical tests defining the properties of each *Aeromonas hydrophila* species within the group were obtained.
In biochemical characterization of isolated strains it was established that they were oxidase positive, catalase positive, arginine positive, ornitin positive, H_2S negative, urease negative, tryptophan desaminase negative, gelatin negative, ferment manithol, inositol, rhamnose and saccharose, they are mobile, hemolytic and hydrolyze starch. In regard to other biochemical properties the following was determined: 11 (91.7%) of 12 studied strains of *Aeromonas hydrophila* have β-galactosidase, also they are citrate and indole positive, sorbitol negative, arabinose positive, produce NO_2 and reduce it to N_2, also that 10 (83.3%) of 12 studied strains were Voges-Proskauer positive and negative to melibiosis, whereas eight (66.7%) of 12 strains had lysine-decarboxilase positive reaction and were amygdalin positive. In 58.3% of strains (seven of 12 strains) it was established that they fermented glucose.

Table 2. Biochemical characteristics of *Aeromons hidrophila* group 2.

Biochemical series		***Aeromonas hydrophila* group 2**				
		Isolated strain number:				
		I	**J**	**K**	**L**	**M**
1.	Presence of β galaktosidase*	+	+	+	+	-
2.	Presence of arginin hidrolase	+	+	+	+	+
3.	Presence of lysin decarboxylase	+	+	+	+	+
4.	Presence of ornitin decarboxilase	-	-	-	-	-
5.	Citrate	+	+	+	+	+
6.	Production of H_2S	-	-	-	-	-
7.	Urease	-	-	-	-	-
8.	Presence of tryptophan desaminase	-	-	-	-	-
9.	Indol	-	+	+	+	+
10.	VP	+	+	+	+	+
11.	Dissolving gelatin	+	+	+	+	+
12.	Presence of citochrome oxidase	+	+	+	+	+
13.	Glucose fermentation*	+	+	-	+	-
14.	Manithol fermentation	+	+	+	+	+
15.	Inositol fermentation	-	-	-	-	-
16.	Sorbitol fermentation	-	-	-	-	-
17.	Rhmanose fermentation	-	-	-	-	-
18.	Saccharose fermentation	+	+	+	+	+
19.	Melibiosis fermentation *	+	+	-	-	-
20.	Amygdalin fermentation *	+	+	-	-	+
21.	Arabinose fermentacija	+	+	+	+	+
22.	TSI	yellow	yellow	yellow	yellow	yellow
23.	Production of NO_2*	+	-	+	+	+
24.	Reduction into gas N_2*	-	+	-	-	-
25.	Mobility	+	+	+	+	+
26.	Growth on McConkey agar	+	+	+	+	+
27.	Hemolysis on blood agar	+	+	+	+	+
28.	m-Aeromonas hydrophila medium	yellow	yellow	yellow	yellow	yellow
29.	Catalase	+	+	+	+	+
30.	OF/ F	+	+	+	+	+
31.	OF / O	-	-	-	-	-

*Note: * Non-specific reactions for group 2*

CONCLUSIONS

Obtained results in regard to biochemical properties of *Aeromonas hydrophila* isolates, isolated from trout tissues, show that phenotype identification schemes for identification of *Aeromonas hydrophila* strains originating from fish at the level of species, should be supplemented. Due to lack of clear phenotype scheme and poor biochemical

characterization of described strains originating from fish, further taxonomic research based on molecular methods are necessary.

ACKNOWLEDGEMENTS

Results were obtained during realization of the project No. TR31011, financed by the Ministry of Education and Science.

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CHEMICAL CHARACTERIZATION OF HULL-LESS PUMPKIN SEED OIL PRESS-CAKE

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ABSTRACT: The utilization of agricultural waste products to produce food has recently received increased attention, not only to minimize waste disposal problems, but to help maximize potential resources and production of new and novel food products. The objective of this study was to characterize the pumpkin oil press-cake, a by-product of the pumpkin seed oil pressing process. The cakes were obtained by pressing the domestic variety of hull-less pumpkin seeds (*Cucurbita pepo* L.) „Olinka". Two pressing processes were applied: using a continual screw press in cold-pressed oil manufacturing and hydraulic press in the virgin pumpkin oil pressing process. In order to assess the suitability of this by-product, proximate analyses of the samples were conducted to determine the residual oil and water contents, as well as proteins, carbohydrates, crude fibers and ash using official methods of analysis. The residual oil was also analyzed in order to determine its nutritive quality. The obtained results showed that two kinds of oil press-cakes were significantly different in terms of oil and water contents. The press-cake obtained using a screw press had higher water content (8.30 ± 0.40%) and lower residual oil content (13.41 ± 1.48%), compared to the press-cake obtained using a hydraulic press (3.94 ± 0.65% water content and 18.18 ± 9.44% residual oil). In addition, the pumpkin press-cakes had a substantial amount of high quality proteins. The protein content of the press-cake obtained using a screw press was 67.09 ± 3.08 % d.m., while the protein content in the cake obtained using a hydraulic press was 59.20 ± 5.13% d.m. Results have also confirmed that the residual oil, regardless of the pressing method used, was of high quality, especially in terms of the high content of the essential omega-6 (linoleic fatty acid) and gamma tocopherols. The obtained results confirmed that both press-cakes can be used in different applications as value-added, functional ingredients for food manufacture.
Key words: *hull-less pumpkin seed, oil press-cake, chemical composition, nutritive value*

INTRODUCTION

There is a huge concern these days over technological processes of raw materials manufacturing that shall be employed in such a way which will provide protection of nutritional components from deterioration. The goal is to have these components in an unchanged form, so the by-products can be fully utilized as high quality ingredients.
The utilization of agricultural waste products to produce food has recently received increased attention, not only to minimize waste disposal problems, but to help maximize potential resources and production of new and novel food products. In this regard it is very important to utilize the oil seeds' press cake obtained using a pressing process, i.e. without using organic solvents, while the press-cake maintains desirable nutritional qualities of raw materials.
Recently reserachers have been paying much attention to nutritional qualities of proteins that originated from the pumpkin seeds, specifically family *Cucurbitaceae* (Ng et al., 2002; Tongyi et al., 2003; Wang and Ng, 2003). Pumpkin seed proteins have pharmacolgical activities such as antifungal (Wang and Ng, 2003), antibacterial and antiinflamatory (Caili et al., 2006), as well as ACE inhibitory and antioxidative properties (Nkosi et al., 2006). It has also been

demonstarted that the addition of pumpkin seed proteins improved the protein digestion of bread (El-Soukkary, 2001).
Due to their high nutritional quality and content of biologically active components, hull-less pumpkin seeds (*Cucurbita pepo* L. var Styriaca and Olinka) have been extensively used in the past decade for oil manufacturing using a pressing process. In some parts of Europe, such as Austria (Fruhwirt and Hermetter, 2008), Slovenia (Kreft et al., 2002), Croatia (Neđeral-Nakić et al., 2006), Germany and Hungary virgin pumpkin oil has been processed for decades, while cold pressed hull-less pumpkin seed oil has been recently manufactured in Serbia, especially Vojvodina. The main difference between the manufacturing processes of these two oils is that cold-pressed oil is obtained by pressing the oil seeds using a screw press at temperatures below 50^{o} C (Vujasinović et al., 2010), while virgin pumpkin seed oil is obtained by pressing thermally processed ground seeds (heated to temperatures of 100-120^{o} C) using hydraulic presses (Vujasinović et al., 2012).
Recent studies have demonstrated that the pumpkin oil press cake has superior nutritional qualities. It is very rich in proteins, essential amino acids, fatty acids (FAs), minerals such as potassium, magnesium, calcium, manganese, zinc, copper, iron, phsphorus, selenium, iodine as well as other bioactive components that are present in the residual press-cake oil (Radočaj et al., 2011).
The objective of this study was to compare the quality of oil press cakes, by-products that are obtained by pressing hull-less pumpkin seeds (*Cucurbita pepo* L.) using different methods for oil recovery.

MATERIAL AND METHODS

Hull-less pumpkin seed oil press-cake samples (domestic variety Olinka) were obtained from a small oil processing company (PANUNION OIL, Novi Sad, Serbia), where the seed pressing process is carried out using a common technology process. 10 press-cake samples obtained using borth, screw and hydraulic presses were investigated. Samples were kept in sealed PE bags until analyzed.
Using a continual screw press „Kern Kraft“ (capacity 40 kg of seeds per h, Germany), where cold-pressed oil was produced, a „*screw press“ oil cake* was obtained. When a hydraulic press (capacity 40 kg of seeds per h, ”Lešnik”, Slovenia) was used for a virgin oil production using ground roasted seeds, a “*hydraulic press” oil cake* was obtained.
Proximate analysis of press cakes was conducted using standard analytical methods: moisture (ISO 665, 2008), oil content (ISO 659, 2007) and crude proteins (SRPS ISO 1871, 1992). Crude fibres and ash were determined using standard methods (AOAC, 1997).
Carbohydrate content was determined by difference.
Oil content of press cakes was determined by Soxhlet extraction (solvent: hexane; 6 hrs.), after which a fatty acid profile and tocopherols content was determined.
Fatty Acid Composition. Fatty acid methyl esters (FAMEs) were prepared according to the standard method [ISO 5509, 2000]. The fatty acid profile was analysed by the gas chromatography method [ISO 5508,1990] on a Hewlett-Packard series II^{plus} GC analyzer equipped with automatic sampler HP 7673, splitless injector, flame-ionization detector and integrator. The gas chromatographic conditions were as follows: a capillary Supelco column SP-2560, 100 m length, injector and detector temperature 220 °C, oven temperature 175 °C, injection volume 1 μL. Helium was used as the carrier gas at a flow rate of 0.9 mL/min. Identification of the individual FAs was accomplished by comparing the GC retention time with that of the pure commercial standard.
Determination of *tocopherols* (Toc) was carried out using high performance liquid chromatography (Waters M600E, United States) on a reversed phase column Nucleosil 50-5 C18 with fluorescence detection (Vujasinović et al., 2012).
Statistical analysis. Data are reported as means ± SD (n = 3). Statistical analysis was performed using Statistica 8 software package. Statistical differences between samples were

estimated by applying one-way ANOVA and using the Tukey test at a significance level of 5% ($p < 0.05$).

RESULTS AND DISCUSSION

Oil and moisture content of samples obtained using a screw press and hydraulic press are presented in tables 1. Proximate analysis of press cakes is presented in table 2.

Table 1. Oil and moisture content of hull-less pumpkin seed oil press cakes obtained using screw and hydraulic presses

Sample	Oil press-cake *screw* press		Oil press-cake *hydraulic* press	
	Oil (%)	Moisture (%)	Oil (%)	Moisture (%)
1	12.1±0.20^{ab}	7.93±0.02^{a}	17.95±0.50^{abc}	5.38±0.03^{a}
2	12.1±0.30^{ab}	8.28±0.04^{b}	14.19±0.30^{de}	3.38±0.06^{b}
3	11.2±0.40^{b}	8.31±0.04^{b}	16.34±0.30^{cf}	3.99±0.04^{cd}
4	15.2±0.20^{cd}	7.63±0.03^{c}	12.69±0.20^{e}	2.99±0.05^{e}
5	16.2±0.50^{d}	8.84±0.05^{d}	18.47±0.40^{ab}	4.01±0.03^{cd}
6	14.8±0.30^{cd}	8.77±0.03^{d}	18.63±0.40^{af}	4.22±0.06 ^{cf}
7	13.5±0.20^{ac}	8.15±0.03^{be}	20.55±0.50^{f}	3.88±0.06^{dg}
8	13.8±0.20^{ac}	8.06±0.02^{ae}	15.01±0.30^{df}	3.68±0.03^{gh}
9	11.0±0.40^{b}	8.80±0.02^{d}	16.64±0.30^{bcf}	4.37±0.03^{f}
10	14.2±0.50^{c}	8.23±0.03^{be}	31.30±0.10^{g}	3.52±0.05^{bh}
Interval	11.0 – 16.2	7.63 – 8.84	12.69 – 31.30	2.99 – 5.38
Mean value	**13.41±1.48**§	**8.30±0.40**§	**18.18±9.44**$^{£}$	**3.94±0.65** $^{£}$

Means within the same column followed by different superscript letters ($^{a-h}$) are significantly different ($p < 0.05$).
Means within the same row followed by different superscript letters ($^{§, £}$) are significantly different ($p < 0.05$).

As it can be seen in table 1, press cake samples obtained using different oil pressing technologies are statistically different in terms of oil and moisture content. The mean value of oil content of the press cakes obtained using a screw press was 13.41 ± 1.48%, where the range of the moisture content of the samples is 11.0 to 16.2%. The mean value of the oil content of the press cakes obtained using a hydraulic press was higher (18.18 ± 9.44%), where the oil content of press cakes was distributed amongst samples in a much higher range (12.69 to 31.30%). The high range in values and differences amongst samples can be explained by the fact that the pressing is a batch process. Besides, the oil pressing process and a quantity of separated oil is dependant on the thermal process (roasting temperature and time) that is performed before the pressing step. Press cake samples are also significantly different (p<0.05) in terms of the moisture content, where the mean values of the press cakes obtanied using these two processes were 8.30 ± 0.40% for the screw press cake and 3.94 ± 0.65% for the hydraulic press cake. It shall be noted here that the moisture content of the screw press cake depends primarily on the moisture content of the raw seeds, while it depends on the thermal process before the pressing in the hydraulic press cake.
Proximate analysis of hull-less pumpkin oil press cakes (table 2) showed that they have very high nutritive values. Besides residual oil, a very high amount of crude proteins (67.09 ± 3.08% d.b.) was found in screw press cakes, as well as in hydraulic press cakes (59.20 ± 5.13%). The carbohydrate content in both types of press cakes was in the range of 9.14 ± 2.16 to 10.82 ± 0.4% (screw and hydraulic press, respectively). Ash content (approx. 6%) indicated a high content of minerals, which is in agreement with a published study (Radočaj et al., 2011). It can be seen that proximate analysis of press cakes obtained using different processes are statistically different, except the crude fibers content.

Table 2. Proximate analysis of hull-less pumpkin press cakes obtained using screw and hydraulic presses

Parameter	Oil press-cake *screw press* [§]	Oil press-cake hydraulic press [£]
Moisture content (%)	8,30±0,40	3,94±0.65
Oil content (% tel quel)	13,41±1,48	18,18±9,44
(% d.b.)	14,62±1,48	18,93±9,44
Crude protein (% tel quel)	61,52±3,08	56,87±5,13
(% d.b.)	67,09±3,08	59,20±5,13
Carbohydrates (%)	9,14±2,16	10,82±0,4
Crude fibers (%)	3,59±0,30*	3,51±0,03*
Ash (%)	5,80±0,50	6,64±0,11

Means within columns followed by different superscript letters ($^{§, £}$) are significantly different ($p < 0,05$) (between the press cakes obtained using screw press and hydraulic press).
**without statistically significant differences*

Nutritional quality of the residual press-cake oils is presented in table 3.

Table 3.Nutritive quality of residual press-cake oils

Parameter	Oil press-cake *screw press*	Oil press-cake *hydraulic press*
Fatty acid composition (% w/w)		
Palmitic acid (C16:0)	13,19±0,11	13,29±0,21
Stearic acid (C18:0)	6,40±0,08	6,74±0,11
Oleic acid (C18:1, ω-9)	26,89±0,62	27,05±0,93
Linoleic acid LA (C18:2, ω-6)	52,13±2,14	51,04±2,05
α-Linolenic acid (C18:3, ω-3)	0,21±0,05	0,23±0,03
Trans fatty acids	0,00	0,17±0,00
Tocopherols (mg/kg):		
α-	16,2±0,6	13,3±0,7
β-	3,4±0,5	4,3±0,5
γ-	777±12,3	704±10,0
δ-	7,84±0,8	1,97±0,5

Results presented in table 3 showed that hull-less pumpkin seed oil (domestic variety Olinka) is of the oleic-linoleic type, with a dominant oleic fatty acid (approx. 27%) and linoleic – omega-6 fatty acid (approx. 52%). In addition, although present in a very small quantity (approx. 0.2%), α-linolenic acid, i.e. omega-3 fatty acid was also detected.
Trans fatty acids were detected in a very small amount (0.17%), only in the hydraulic press cake, which is a cake obtained in the pumpkin virgin oil production process. Currently, there is a demand in the market for food products that are sources of omega-3 and omega-6 fatty acids.The omega-3 and omega-6 fatty acids are essential fatty acids, necessary from conception through pregnancy and infancy, and throughout life in the prevention of certain diseases (Connor, 2000). Omega-6 fatty acids are considered to increase lipid peroxidation, while the omega-3 FAs exert a chemo preventive effect (De Barros et al., 2011).
Tocopherols, as very important minor components of an oil with high antioxidant activity, were also found in significant amounts in the residual oil present in all cakes. The predominant type of tocopherols was the gamma tocopherol in the amount of 700 mg/kg oil. Both, fatty acid profile and tocopherol contents contribute significantly to the increase of the nutritive quality of the press cakes.
As it can be seen in table 3, residual press cake oils have a very similar fatty acid profile. However, their tocopherol profile is different and higher values were obtained using a screw press. In respect to such nutritive characteristics, hull-less pumpkin oil press cake can be used for the development of value-added, functional food products such as a fat based spread.

CONCLUSIONS

Obtained results have shown that the hull-less pumpkin seed oil press-cake which is a by-product of either the screw or hydraulic pressing process, has a very good chemical and nutritional quality, and is a suitable raw material for the manufacturing of different food products.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Education and Science, Republic of Serbia, for the grant (project: TR 31014, Development of the new functional confectionery products based on oil crops) and wish to thank Branislav Panić from the Oil mill plant PANUNION OIL Novi Sad for the supply of press-cake samples.

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CHEMICAL COMPOSITION OF MULTIFLORAL BEE POLLEN FROM BULGARIA

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ABSTRACT: Bee pollen is promoted as a healthy food with a wide range of nutritional and therapeutic properties. The objective of the present study is to determine the chemical composition of multifloral bee pollen produced in Bulgaria. The study was carried out totally on fourteen bee pollen samples. The following parameters were determined: Moisture content – until constant weight, using oven at 105 ºC for two days; proteins by Kjeldahl method; lipids by Soxhlet procedure using diethyl ether as solvent; ash content – gravimetry using oven at 550 ^{o}C. Before testing the samples are stored in a refrigerator at -18 ºC. Ten commercial bee pollen samples were purchased from the market. It was found that bee pollen contains: moisture (8.92 – 14.60%), proteins (22.05 – 25.96%), lipids (3.81 – 9.32%) ash (2.30 – 2.80%). Four samples came from the experimental apiaries of Institute of Animal Science (IAS), Kostinbrod. For these samples the following results were obtained: moisture (11.39 – 16.48%), proteins (18.45 – 22.42 %), lipids (6.30 – 8.71%), ash (1.56 – 2.22%). The average values of moisture (13.81% ± 1.06), protein (19.80% ± 0.89), lipid (7.15% ± 0.54) and ash content (1.81% ± 0.14) of bee pollen samples collected at IAS are comparable with those of commercial samples – 11.51% ± 0.55; 23.48% ± 0.44; 6.38% ± 0.54; 2.58% ± 0.06, respectively. The variation between the samples can be explained by multifloral origin.
Key words: *pollen, chemical composition, moisture, proteins, lipids, ash.*

INTRODUCTION

Bee pollen is promoted as a healthy food with a wide range of nutritional and therapeutic properties. A high concentration of proteins, essential amino acids, sugars, unsaturated and saturated fatty acids, the presence of essential elements and vitamins (Almeida-Muradian et al., 2005; Villanueva et al., 2002) make bee pollen very important for human diets. These components depend on the botanical origin of pollen and variation in absolute amounts of the different compounds can be very high. Regardless of these proven benefits to human health, little attention has been paid to the development of methods for assessing the quality and composition of bee pollen.

Honey bees *Apis Mellifera* feed mainly on nectar and pollen. Pollen is the main source of protein for the bee colony. Pollen is produced in various countries as a feeding supplement for human nutrition.

The major components in bee pollen are proteins and amino acid, lipids (fats or their derivatives) and sugars.

Protein content is a standard determination of % N using the Kjedahl method, using a conversion factor of 6.25 (Rabie et al, 1983). The predominant amino acids are proline, glutamic and aspartic acids, lysine and leucine.

Analysis of pollens generally indicates that they are composed of about 50% polysaccharide, 4–10% simple sugars, 1–20% lipids, 6–28% protein, and 6% amino acids, accompanied by a variety of secondary plant products such as flavonoids, c arotenoids, terpenes (Tyler, 1993).

Protein contents of above 40% have been reported, but the typical range is 7.5 to 35%: sugar content ranges from 15 to 50% and starch content is very high (up to 18%) in some wind-pollinated grasses (Schmidt and Buchmann, 1992).

According to Campos et al. (2008) proteins are between 10 – 40%, lipids 1 – 13%, ash 2 – 6%.

The objective of the present study is to determine the chemical composition of multifloral bee pollen produced in Bulgaria, including moisture, proteins, lipids and ash content.

MATERIAL AND METHODS

The study was carried out at Institute of Animal Science (IAS), Kostinbrod, Bulgaria. Fourteen multifloral bee pollen samples were investigated. Four freshly bee-collected samples were harvested during two months (June and July) in 2010 from the experimental apiaries of IAS and ten samples were purchased from the market. Once collected, the samples from IAS were immediately dried at 30 °C. All pollen samples were kept frozen (-18 °C) in a refrigerator until analysis.
The proximate composition of the pollen was determined by using standard methods of food analysis:
Moisture content was made through gravimetry until constant weight, using oven at 60 ºC for three days and at 105 ºC for two days (sample of 5 g). Dry matter of the samples was obtained by subtracting the moisture content from 100.
Protein content was obtained using Kjeldahl method (sample of 2 g).
Lipid content was determined by Soxhlet extractor using diethyl ether as solvent (sample of 5 g).
Ash content was determined by gravimetry using oven at 550 ºC until constant weight (sample of 2 g).
All analyses were done in duplicate. Statistical analysis was performed using MS Excel 2007.

RESULTS AND DISCUSSION

The chemical composition of LRJ samples are presented in Table 1, Table 2 and Figure 1.

Table 1. Moisture, dry matter, proteins, lipids and ash of bee pollen from IAS

	Mean±S.E	**Min**	**Max**
Contents			
Moisture, %	13.81±1.06	11.39	16.48
Dry matter, %	86.19±1.06	83.52	88.61
Proteins, %	19.80±0.89	18.45	22.42
Lipids, %	7.15±0.54	6.30	8.71
Ash, %	1.81±0.14	1.56	2.22

Results are given as minimum, maximum, mean ± standard error (n = 4)

Table 2. Moisture, dry matter, proteins, lipids and ash of bee pollen purchased from the market

	Mean±S.E	**Min**	**Max**
Contents			
Moisture, %	11.51±0.55	8.92	14.60
Dry matter, %	88.49±0.55	85.40	91.08
Proteins, %	23.48±0.44	22.05	25.96
Lipids, %	6.38±0.54	3.81	9.32
Ash, %	2.58±0.06	2.30	2.80

Results are given as minimum, maximum, mean ± standard error (n = 10)

Moisture content of the tested samples ranged from 11.39 to 16.48% for the samples from IAS (Table 1) and from 8.92 to 14.60% for the samples from the market (Table 2). The average content of that constituent was also higher in the pollen samples from IAS – 13.81%. The product submitted to a drying out process in temperatures not higher than 30 °C. The higher temperature led to significant losses of vitamin and enzymes.
The content of pollen total protein in the samples from the market was higher (22.05 – 25.96%) than the other samples (18.45 – 22.42%). The protein content of pollen depends strongly on the botanical origin.
The comparison of the lipid values obtained in our samples with the samples from the market showed that the highest lipid concentration was predominant in the purchased samples

(3.81 – 9.32%). This fact could be explained with the high variation of this index and these values are comparable with the lipid levels reported in the literature (Campos et al., 2008).
As can be seen in Table 1 and Table 2 the ash content and the concentration of mineral elements do not exhibit a large range in the both groups of samples. There is considerable variation depending on the pollen type. According to Szczesna (2007) ash content is between 2.08 – 3.19%.

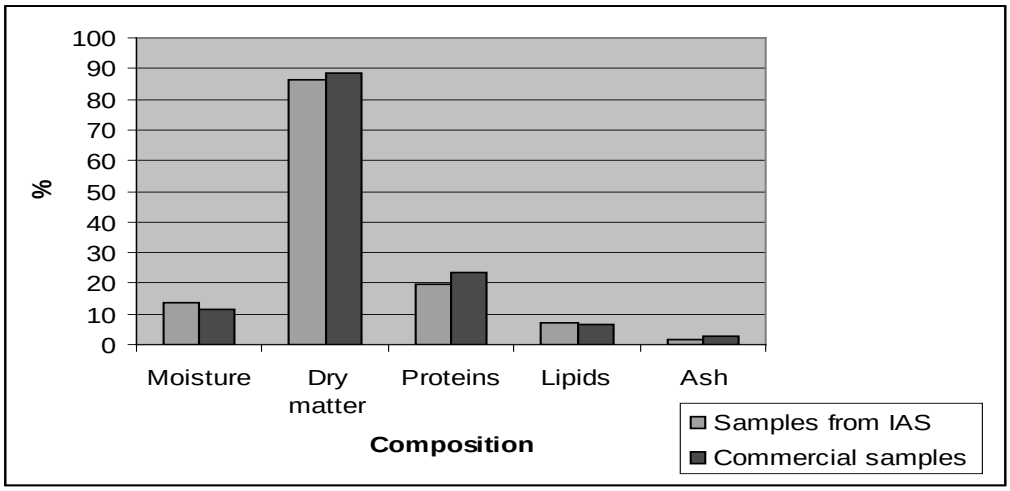

Figure 1. Chemical composition of bee pollen

CONCLUSIONS

The average values of moisture (13.81% ± 1.06), protein (19.80% ± 0.89), lipid (7.15% ± 0.54) and ash content (1.8 % ± 0.14) of bee pollen samples collected at IAS are comparable with those of commercial samples – 11.51% ± 0.55; 23.48% ± 0.44; 6.38% ± 0.54; 2.58% ± 0.06, respectively. Protein and ash contents were slightly higher in the purchased samples while the lipid content was lower. The variation between the samples can be explained by multifloral origin.

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CONTENT OF HEAVY METALS IN CARPOPHORES OF WILD MUSHROOM (*Boletus edulis*)

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ABSTRACT: *Boletus edulis* is among the most popular and widely consumed wild macromycetes, being rich in minerals, dietary fiber, vitamins and having medical properties they have been used as antibacterial, anti-tumor, anti-cholesterol and antiviral agents. This research was conducted on 9 areas of SouthWest Serbia covering forests. Results of the research showed the main soil characteristics to be as follows: pH values ranging from 3.50 to 6.75, total organic carbon – 1.65-6.84%; and total humus range - 2.83-10.75%. Total content of heavy metals detected in all examined soil samples was under the permitted values, as well as in all the examined stone fungus samples, with the difference in Cd content ranging from 0.08 to 1.3 mg/kg. One of the tested localitites had exceeding permitted values of Cd content (>1mg/kg), as well as Hg content showing to be above the permitted values on 3 localities (0.58-0.71 mg/kg). These results indicate the absorption capacity of the wild mushroom to be higher for As and Pb, comparing to the present Cd from the soil. It is also interesting for Hg to be detectable in the mushroom samples deriving from the soils without Hg content, which indicates further invastigations regarding potential pollution sources.
Key words: *wild mushroom, heavy metals, absorption capacity*

INTRODUCTION

Mushrooms are important part of human diet. Furthermore, export of mushrooms is important part of total agricultural products that are being exported from Republic of Serbia to EU countries and Russian Federation. For all these reasons it is necessary to control the heavy metal content in mushrooms in order to provide safe food and environment protection. Mushrooms are considered to be healthy food with higher content of proteins and carbohydrates than vegetables.They present wide source of biologically active compounds and minerals (Mattila et al., 2001). Furthermore, mushrooms have certain medical properties, as well as antibacterial, anti cancer, antiviral influence and play important role in decreasing cholesterol content (Cochran, 1978; Chobot et al, 1997; Mattila et al 2000). Some researches showed that mushrooms have antiallergenic effect and can be used preventively against cholesterol, tumor and cancer (Visser, 1993).
The absorption and accumulation of heavy metals is dependent on mushroom species, growing substrate, age of mycelia and interval between mushroom appearance (Kalac and Svoboda, 2000).
Studies of heavy metal content in mushrooms showed a correlation between heavy metal concentration and point sources of contamination, as furnace and nearness of roads where the mushrooms are collected (Bargagli and Baldi, 1984; Isildak et al., 2004).
Considering the vast presence of mushrooms in forest ecosystems, metal content in forest soils is important parameter for mushrooms abundance, yield and biological value.
Some metals (Cd, Pb, Cr and Zn) are concentrated mostly in mushroom cap. Mushrooms collected near urban settlements have higher content of heavy metals (Cd, Cr, Ni, Pb) Therefore the determination of heavy metals in Boletus (and other edible mushrooms) is important parameter of safety and human health. For these reasons, determination of health

safety of Boletus (and other edible mushrooms) needs identification of potential sources of contamination on locations where mushrooms are collected.
Unlike As, Cd, Pb, which are present in all geological substrates and soil profiles, Hg is sporadically present in some location, but because of its high toxicity it is necessary to monitor its content. Highest level of some pollutants in foodstuffs, according to Board of European committee (EC 466/2001) is 0.2 and 0.3 mg/kg wet mass for Cd and Pb.
World Health Organization declares that weekly input of Cd and Pb for adults through food and agricultural products is between 0.42-0.49 and 1.5 mg, respectively. Content of 1.75 mg is considered to be highest level allowed (Leski and Rudawska, 2005).
The aim of this paper was to determine the relationship between heavy metals content in mushrooms and its content in substrate (forest soil).

MATERIAL AND METHODS

The wild edible mushroom *(Boletus edulis)* and soil samples (0-10 cm deeper) simultaneously were collected from nine, mostly forest location in SouthWest Serbia area following location and numbers (in tables): municipality of Priboj (Sjeverin, No. 1); Municipality Prijepolje (Jabuka-fagus forest, No.2., Jabuka-juniper bush, No.3.; Hisarđžik and i Kosatnica, No.5.; Prevoj and Drmanovići No. 6.; Aljinovići No. 7.), Brodarevo (No. 4.), Municipality Sjenica (Štavalj No. 8. Sugubine, No. 9.). Each location was divided in 3 sublocation, from which the mushroom samples were taken and analysed for heavy metal content. On the main mountain locations soil and *Boletus edulis* were paralely sampled.
The main soil characteristics (humus, pH values and organic C) have been analyzed using standard methods (electrochemicaly by nKCl, Kotzman method and CNS-analyzer), and the content of heavy metals As (detection limit 0.02), Cd (detection limit 0.1), Pb (detection limit 0.25) were determined using ICP technique. Atomic absorption spectroscopy (AAS) employing hydrid metod was used to determine the content of Hg in the examined soils (detection limit 0.02). In order to analyze the content of heavy metals in mushrooms, there has been used AAS method.

RESULTS AND DISCUSSION

Heavy metals have negative effects on all biological processes in plants as well as on mineral nutrition and biochemistry of wild mushrooms (Udosen et al., 2001). Generally metal absorptions in mushrooms (as well as in plants) depend on climate, rains, forest horizons, age of mycelium, time interval among mushrooms appearance and many other factors (Kalac and Svoboda, 2000). The main soil characteristics and content of heavy metals are presented in Table 1. Average pH values in soils were from 3.50 to 6.75. Content of organic C was ranging from 1.65 to 6.84%, and humus content was from 2.83 to10.75%. In most of the examined forest soils the pH was in acidic range, and only 1 sample had neutral pH values. For metal absorption these data are very important, since the acid soils had higher concentration of metal ions that can be readily absorbed by plants and mushrooms.
The content of As at investigated localities showed high degree of variation from 0.075 to 7.94 mg/kg. Pb content ranged from 14.06 to 69.85 mg/kg, although variation were not high at 8 investigated localities, ranging from 14.06 to 34.31 mg/kg. The Cd content in soil was found to be from 0.39 to 2.11 mg/kg. The Hg content in soil was not found in 7 localities except localities 4 (0.06) and 6 (0.018 mg/kg as presented in Table 1. According to limits allowed by Serbian law, contents of heavy metals in all the investigated samples were under the limits.

Table. 1. Heavy metal contents and soil characteristic of forest soil at Serbia Southwest area.

Location No.	Soil heavy metal contents (mg/kg)				Soil characteristics		
	As	**Pb**	**Cd**	**Hg**	**pH (nKCl)**	**Org.C %**	**Humus %**
1.	4.94	14.06	0.67	*	3.60	1.94	3.35
2.	7.94	17.88	1.03	*	4.30	1.65	2.83
3.	3.53	14.91	0.39	*	3.50	3.82	5.00
4.	6.07	69.85	2.11	0.06	6.75	6.53	10.75
5.	2.66	20.2	0.60	*	3.70	6.84	9.63
6.	3.35	34.31	0.71	0.018	3.65	2.90	4.98
7.	0.075	16.46	0.69	*	3.90	4.27	6.48
8.	4.32	22.47	0.46	*	3.80	3.54	5.69
9.	7.48	25.77	1.49	*	5.40	5.45	9.30

** - under detection limit*

Table 2. Heavy metals contents (mg/kg) in *Boletus edulis*

Location No.	Heavy metal contents in mushroom (interval)			
	As	Pb	Cd	Hg
1.	*	*	0.08-0.30	0.27-0.58
2.	*	*	0.26-0.40	0.42-0.71
3.	*	*	0.27-0.33	0.14-0.58
4.	*	*	0.14-0.18	0.10-0.37
5.	*	*	0.10-0.16	0.23-0.33
6.	*	*	0.70-1.30	0.14-0.45
7.	*	*	0.36-0.70	0.14-0.41
8.	*	*	0.20-0.48	0.10-0.20
9.	*	*	0.24-0.40	0.10-0.12

** - under detection limit*

According to the results from Table 2 only As and Pb were under detection limits. However content of Cd and Hg in most samples were higher than the limits given by Serbian law. The Cd content variation were from 0.08 to 1.30 mg/kg, and only in area No. 6 the content was higher than allowed (>1mg/kg). These results are similar to results from other research (Jilmaz et al., 1998; Turkekul et al., 2004). Opposite to low Hg content in forest soil (4 and 6 localities), the Hg contents in mushrooms were 0.10 to 0.71 mg/kg, and at 3 localities the Hg contents were higher that it allowed (>0.5 mg/kg). These results were similar to the results of Falandzysz et al. (2007).

CONCLUSIONS

The content of heavy metals in soils was found to be in most samples and localities under the allowed limits by Serbian law. The results showed also that As and Pb were not absorbed by mushrooms. However the Cd and Hg content in mushrooms were higher comparing to soil samples. However, the Hg was found in mushrooms grown on the soils where there are no genuine presences of Hg. This implies further research in this area, since the given results do not indicate the origin of Hg pollution.

ACKNOWLEDGEMENTS

This research was partially supported by Ministry of education and science of Serbia (grant number TR31080).

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EVALUATION OF BROWNING RATE OF QUINCE AT AMBIENT EXPOSURE

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ABSTRACT: Since quince is a fruit relatively susceptible to browning, in the present work it was evaluated the colour of the quince over time of exposure to the atmospheric air. The colour of the fresh pulp was assessed using a handheld tristimulus colorimeter using the CIELab colour coordinates: L*a*b*. These Cartesian coordinates were then used to calculate the polar or cylindrical coordinates: value, hue angle (º) and chroma. At each instant, ten measurements of colour were made, and to evaluate the colour change, measurements were repeated in the same samples after every 5 minutes, over a period of two hours. The results obtained for the medium values of the cylindrical coordinates in the freshly cut quince were found to be: value = 7.80, chroma = 31.78 and Hue = 92.28º, being these values determined right after cutting. Total colour difference (TCD) was calculated having the values of the Cartesian coordinates of the freshly cut quince as reference. In relation to the colour change, the values for TCD were 0.00, 26.35, 30.41, 31.81 and 32.39, respectively for times 0, 30, 60, 90 and 120 minutes, indicating a faster rate initially and a tendency for stabilization towards the end of the period analyzed.
Key words: *quince, browning, colour, browning kinetics*

INTRODUCTION

The Quince (*Cydonia oblonga*) is a relatively small tree, the only member of the gender *Cydonia*, from the Rosaceae family. The fruit is called quince, and resembles a cross between an apple and a pear. Quince is rich in dietary fibre, vitamins, like vitamin C, and minerals, such as copper or potassium, while being low in saturated fat, cholesterol and sodium. In the raw form, the flesh is hard and unpalatable, with an astringent, acidulous taste. However, quince is rarely used in the raw form, being instead commonly made into preserves and jellies. Once cooked and sweetened, it turns red, with a pleasant taste and strong fragrance.

Colour is one of the most important appearance attributes of foods and it has a major influence on the reaction of the consumer, contributing to determine the acceptability or rejection of the food. However, the colour of food products is quite susceptible, and may be affected in a high extent by processing, in particular thermal processing. Nevertheless, some degradation also occurs during transportation and storage. Among the alterations that can occur are those resulting from pigment degradation, browning reactions like Maillard reactions, enzymatic browning or oxidation of ascorbic acid (Maskan, 2001, 2006; Suh et al., 2003).

Because colour is such an important attribute of food quality, it has been the object of study by many researchers, in many foods: pomegranate juice concentrate (Maskan, 2006), tofu (Baik and Mittal, 2003), kiwi (Maskan, 2001), spinach (Nisha et al., 2004), watercress (Cruz et al., 2007), carrots (Koca et al., 2007), bananas (Chua et al., 2001) or pears (Quevedo et al., 2009).

Tristimulus colorimetry has been widely accepted as a rapid and simple instrumental method for measuring the colour of food products, and CIELab Cartesian coordinates (L*, a*, b*) can be used to quantify that colour. There are other parameters derived from Hunter L*, a*, b* coordinates, such as the total colour difference (TCD), which aims at quantifying the overall colour difference between a sample and a reference material. Furthermore, the Cartesian coordinates can be used to calculate the cylindrical coordinates: value, chroma and Hue

angle (Maskan, 2001). Data about the kinetics of change in quality attributes in foods, like for example colour, provide valuable information for understanding and predicting changes that occur during processing and storage, and thus allow improving quality and minimizing losses (Kumar et al., 2006).
Although many studies can be found about the kinetics of colour change during processing of foods, studies about the kinetics of degradation of colour of quince just by exposure to the atmospheric air are lacking, and this is a product that is very susceptible to browning and quite rapidly too. In this way, the present study aimed at investigating the variation along time of colour of quince during exposure to atmospheric conditions.

MATERIAL AND METHODS

Sampling

The samples of quince (*Cydonia oblonga* Mill.) cultivar Gamboa were cut into slices and colour measurements were done right after cutting. After that, the slices were left exposed to atmospheric conditions, and colour measurements were made every five minutes until two hours.

Colour measurements

The colour was measured using a handheld tristimulus colorimeter (Chroma Meter - CR-400, Konica Minolta), calibrated with a CIE standard illuminant D65. The colour coordinates L*a*b* of the CIELab colour space were determined, where L* denotes lightness, and varies from zero (black) to 100 (white), a* varies from -60 (green) to +60 (red) and b* veries from -60 (blue) to +60 (yellow) (Kumar et al., 2006). From the Cartesian coordinates (L*a*b*) the total colour difference (TCD) was calculated by equation (1) (Chen and Ramaswamy, 2002):

$$\mathrm{TCD} = \sqrt{(\mathrm{L}^*_0 - \mathrm{L}^*)^2 + (\mathrm{a}^*_0 - \mathrm{a}^*)^2 + (\mathrm{b}^*_0 - \mathrm{b}^*)^2} \quad (1)$$

considering the colour of the freshly cut product as reference. The Cartesian colour coordinates were then used to calculate the cylindrical coordinates, value, chroma and hue angle, by the following equations (Maskan, 2001):

$$\mathrm{Value} = \mathrm{L}^*/10 \quad (2)$$

$$\mathrm{Chroma} = \sqrt{\mathrm{a}^{*2} + \mathrm{b}^{*2}} \quad (3)$$

$$\mathrm{Hue}\,(^{\circ}) = \tan^{-1}\left(\frac{\mathrm{b}^*}{\mathrm{a}^*}\right) \quad (4)$$

According to Maskan (2001) the browning index (BI) can be calculated from the Cartesian colour coordinates by the following equation:

$$\mathrm{BI} = \frac{\left[100\,(\mathrm{x} - 0.31)\right]}{0.17} \quad (5)$$

where

$$\mathrm{x} = \frac{(\mathrm{a}^* + 1.75\,\mathrm{L}^*)}{(5.645\,\mathrm{L}^* + \mathrm{a}^* - 3.012\,\mathrm{b}^*)} \quad (6)$$

For the colour determinations ten samples were analysed at each time instant and the mean values and standard deviations were calculated for each set.

Mathematical modelling

One of the many models cited in literature to describe the kinetics of change of a certain attribute, P, of a food product is the logistic model reported by Chen and Ramaswamy (2002) as:

$$\mathrm{P} = \mathrm{U}_0 + \frac{\mathrm{U}_e}{1 + \exp\left[\pm\,\mathrm{k}(\mathrm{t} - \mathrm{t}_0)\right]} \quad (7)$$

where k is the kinetic constant, U_0 and U_e are constants related to P_0 and P_e, respectively, and t_0 is the half-life time, i.e., the time at which the value of the property P

increases/decreases to half/double of the U value. P_0 represents the initial value of the property while P_e is the final equilibrium value, and the signs (+) or (-) indicate increase or degradation of the property P, depending on the case.

RESULTS AND DISCUSSION

Table 1 shows the results of the fittings made with the logistic model in Equation (7) to the experimental data of the different variables analysed (Cartesian colour coordinates, cylindrical coordinates, total colour difference and browning index). For the fittings, the software SigmaPlot V8.0 (SPSS, Inc.) was used. In Table 1 the values of all four parameters in the model are presented, along with the value of the correlation coefficient, R, which allows evaluating the quality of the fit, i.e., how good does the fit describe the experimental set of data for each case. The values for R in table 1 indicate that in all cases the model adjusts quite well the experimental data, being the fit for a* the best, with higher value of R, 0.9968, and the fit for b* the less good, with the lowest value, 0.9282. Still, since the value of R is quite close to 1, even in this case the fitting was quite successful. The values of the kinetic constant, k, are positive for L* and Hue and negative for all other cases, thus indicating that the variables L* and Hue decrease with time, whereas all other increase.

Table 1. Fitting of the experimental data with the kinetic model from Equation (7)

	L*	a*	b*	Chroma	Hue	TCD	BI
U_0	54.6055	-21.421	29.1377	27.3189	290.72	-36.138	15.2884
U_e	58.1742	39.1257	10.9047	16.27523	-1020.1	67.1939	124.178
t_0	-325.23	-25.239	317.35	402.94	-2328.8	-96.508	626.87
k (s^{-1})	1.04e-3	-1.29e-3	-3.55e-3	-2.34e-3	65.781	-1.25e-3	-1.58e-3
R	0.9918	0.9968	0.9282	0.9784	0.9963	0.9953	0.9920

To better understand how these different colour variables change along exposure time, Figures 1 to 4 show the experimental points of the variables along time, together with the corresponding fits obtained with SigmaPlot. In all cases, each point at a given instant results from the data measured in that instant, i.e., is the mean value between 10 measurements.

Figure 1 shows the different Cartesian colour coordinates, lightness (L*) and the two opposing colour coordinates (a* and b*). The graph evidences the decrease in L* along time, thus indicating that the quince samples become darker. As to the colour coordinate a* the freshly cut quince presents a value for a* lower than zero, thus indicating a slight prevalence of the green colour. However, very rapidly this vanishes giving place to positive values of a*, corresponding to the appearance of the reddish colour, as a result of the development of the brown compounds. As to b*, this is always positive, thus indicating that yellow prevails over blue (negative values of b*), and by increasing with time it shows that the yellowish colour is intensified, this also in result of browning. Furthermore, it is visible from all curves that colour change is faster at the initial moments (until 2000 sec, that is the first half hour), tending to stabilize thereafter.

Figure 2 shows the cylindrical colour coordinates as well as the corresponding fits obtained for each variable. Chroma, which indicates colour saturation, increases along exposure time, thus meaning that the colour is becoming more intense. The hue angle, which is used to characterise the colour of foods, diminishes from around 90º to about 70º. An angle of 0° or 360° represents red Hue, while angles of 90°, 180° and 270° represent yellow, green and blue Hue, respectively.

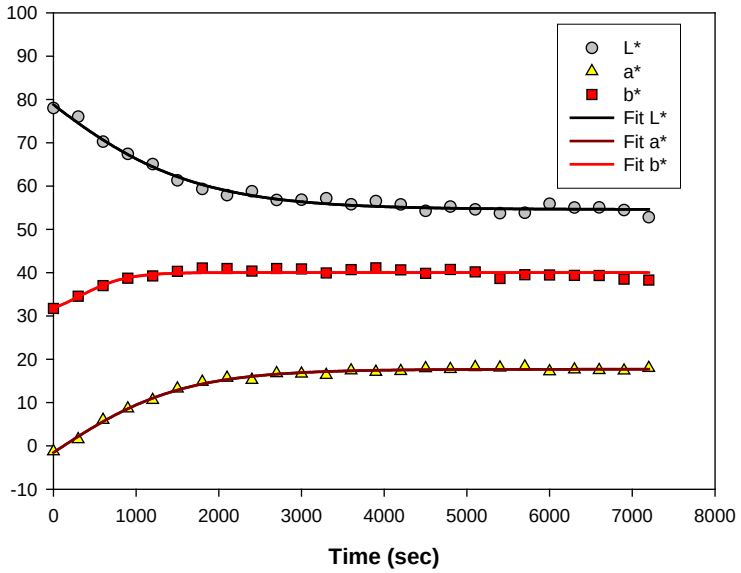

Figure 1. Variation along time of the Cartesian colour coordinates of quince

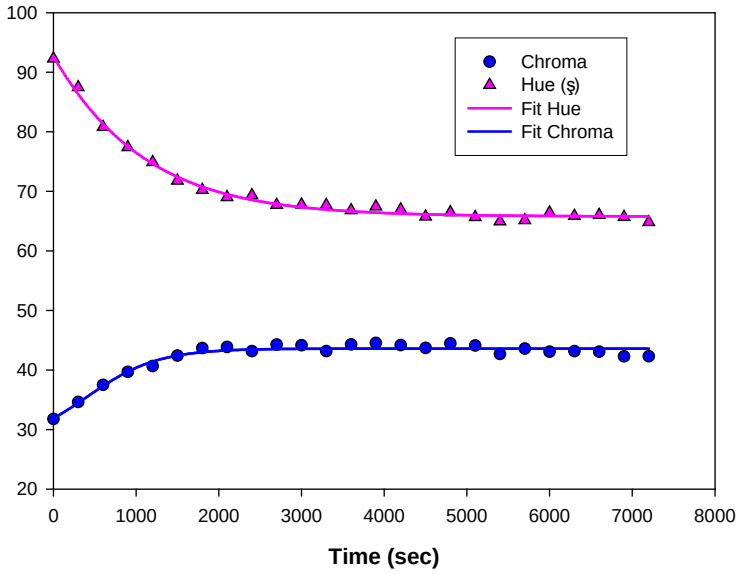

Figure 2. Variation along time of the polar colour coordinates of quince

Figure 3 shows the variation along exposure time of the total colour difference, which was calculated according to Equation (1), and that quantifies the deviation of colour in relation to the reference colour. In this case the freshly cut product was the reference and the corresponding Cartesian coordinates were: L* = 78.02(±0.86), a* = -1.27(±0.23) and b* = 31.75(±1.68). As the graph shows, the values of TCD increase along time, thus indicating a higher difference from the reference, i.e., from the freshly cut quince. This results, as previously said, from brown compounds formed during reactions that take place at the surface of the product that is exposed to the atmospheric air, and in particular oxygen.

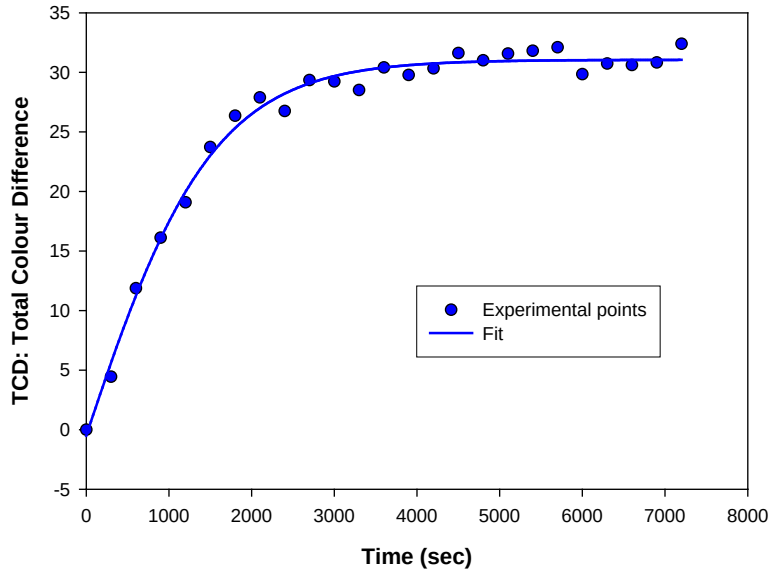

Figure 3. Variation along time of the total colour difference of quince

Figure 4, which represents the variation of the browning index along exposure time, shows a quite similar trend to that observed earlier in Figure 3. In fact, these two variables are different ways of quantifying the same type of phenomena, i.e., the development of darker colours as a result of browning. Once again is visible that the first half hour is critical for the darkening of the cut quince, and that in fact the degree of browning is quite important, given the high values of BI obtained towards the end reaching 140.

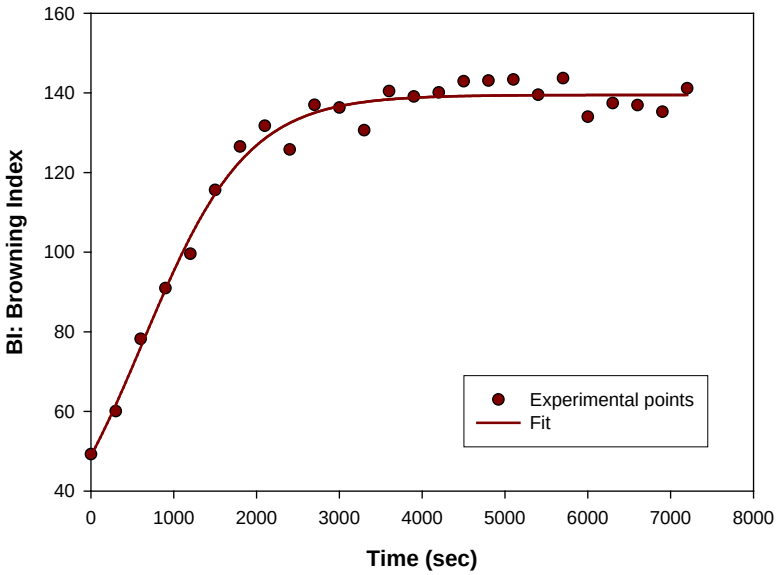

Figure 4. Variation along time of the browning index of quince

CONCLUSIONS

From the present work it was possible to confirm that quince is in fact very susceptible to the exposure to the atmospheric oxygen, which allows the development of an intense degree of browning. All the colour parameters analysed prove the appearance of brown compounds in result of the reactions undertaken at the surface of the quince. Furthermore, total colour difference and browning index were quantified and they revealed that the major colour

deterioration happens in the first half hour, and in the following 1 hour and a half the colour stays approximately constant. Furthermore, the experimental data were fitted to a kinetic model found in literature to describe browning in foods, the logistic model, and in all cases this proved to be very adequate to describe the kinetic behaviour of all variables along time, given the very high values of the regression coefficients.

ACKNOWLEDGEMENTS

The authors thank the financial support to the research centres CI&DETS (Viseu) and CERNAS (Coimbra).

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DETERMINATION OF POLYCHLORINATED BIPHENYLS IN HUMAN COLOSTRUM AND INFANT FORMULAS

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ABSTRACT: Monitoring of PCBs in human colostrum in South Bačka started in 1982. The objective of this study was to determine concentrations of eight EPA PCB congeners (28, 52, 101, 118, 138, 153, 180 and 209) as typical representatives of persistent pollutants, in early human milk and commercial infant formulas, as a measure of exposure of breast fed newborn babies. Samples of 3rd day colostrum were collected in 2006 from mothers living in the region of Novi Sad, who had no known occupational or dietary exposure to chemicals, and analyzed on GC-ECD (HP 5890 supplied with a Quadrex fused silica column 5% Ph for PCBs). The age of mothers, donors of colostrum, was 28.73 ± 2.45 (25 – 32) years - nine were primiparous and thirteen multiparous. The weight of mothers before pregnancy was 61.5 ± 10.2 kg (median 62, range 51 – 87) and before delivery 77.9 ± 10.9 kg (median 80, range 67 – 102).
The sum of 8 PCB congeners was 1.06 ± 1.41 μg/kg wet weight (range 0.15 – 5.60). The highest values were of PCB 101. PCB congeners 28 and 118 were not detected in any of the samples analyzed. No correlation was found between PCBs concentrations and age of mothers. Birth weights also did not influence PCBs concentrations. Concentrations of PCBs in infant formulas were below average values in human colostrum. Formula manufactured in Serbia did not contain any of PCB congeners. In other four imported formulas, PCB congeners 153, 138 and 180 were detected in a concentration ranging from 0.05 to 0.07 ng/g wet weights. Although breast fed babies were more exposed to PCBs than formula fed babies, breastfeeding should be encouraged, since human milk is undoubtedly the best food for development and growth of infants.
Key words: *EPA congeners, human milk, infant formula*

INTRODUCTION

PCBs are persistent in the environment, and their high lipophilicity results in their bioaccumulation and bioconcentration into the biota and biomagnification through the food chain. Bioaccumulation factors of PCBs increase with higher chlorination and lower water solubility (Coristine et al., 1996). Bioconcentration factors (BCFs) for five chlorinated PCB 118 was measured to be 3.60 - 5.19, while for six chlorinated PCB 138 and PCB 153 were measured in range 4.79 - 5.96 and 3.31 - 5.57,respectively for dry weight of various marine species (Hope et al., 1998).
Depuration for less chlorinated PCBs (28, 52, 101) was presented with half time of 44 to 65 days in 30 d dietary exposure followed by 160 d depuration studies, while for highly chlorinated PCBs (101, 118, 138) elimination half time was from 56 to 224 d (Fisk et al., 1998). Less chlorinated PCBs (1–4 chlorines) are readily taken up by organisms, but are readily eliminated and metabolized. Thus, these homologs are not bioaccumulated to a great extent. The most highly chlorinated congeners (7–10 chlorines) occur in low concentrations in the environment, and are tightly bound with soil, sediment, and organic matter.
Most PCBs are slightly soluble in water and the solubility decreases with chorine content. Estimates of PCB solubility range from 2.7 to 15000 μg/l. PCBs have a high affinity for suspended solids, especially those higher in organic carbon. This is supported by their low

water solubility and high octanol/water partition coefficients (calculated Log Kow values range from 3.76 for biphenyl to 8.26 for decachlorobiphenyl).
PCBs, which have log Kow values >5, appear to enter biota through food-web transfer from sediment, which is less efficient. On the other hand, the penta-, hexa-, and hepta-PCBs are both bioavailable and resistant to degradation in organisms; and these PCB homologs bioaccumulate in organisms to the greatest extent (Willman et al., 1997). For example, the PCBs that dominate congener profiles in the tissues of mussels, crabs, and seals are hexa-PCB isomers 138 and 153 (Porte and Albaiges, 1993). Thus, these persistent lipophilic environmental contaminants accumulate in the human body. The elimination of such compounds and their lipophilic metabolites from body fat is very low. Due to the relatively high fat content of breast milk, the lipophilic compounds are transferred to the milk.
Breast milk is a convenient matrix for monitoring persistent organic pollutants (POPs) in human and for studies of time related trends in environmental contamination. The advantages include simple and non-invasive sample collection, suitability for determination of lipophilic POPs due to the relatively rich lipid content, and relevance with regard to the exposure of breastfeeding infants, who are at the early stage of development and vulnerable to toxic contaminants. However, due to the transfer of the mother’s body burden to the infant (Trapp et al., 2008), the contamination levels in female donors may vary with lactation time. Indeed, the levels of POPs in mother’s milk have been reported to decrease with the number of children (Kunisue et al., 2006; Sudaryanto et al., 2006).
Infant formula is a manufactured food designed and marketed for feeding to babies and infants under 12 months of age, usually prepared for bottle-feeding or cup-feeding from powder (mixed with water) or liquid (with or without additional water). The U.S. Federal Food, Drug, and Cosmetic Act (FFDCA) defines infant formula as "a food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk".
The composition of infant formula is designed to be roughly based on a mother's milk at approximately one to three through breastfeeding months postpartum, although there are still significant differences in the nutrient content of these products. The most commonly used infant formulas contain purified cow's milk whey and casein as a protein source, a blend of vegetable oils as a fat source, lactose as a carbohydrate source, a vitamin-mineral mix, and other ingredients depending on the manufacturer. In addition, there are infant formulas using soybean as a protein source in place of cow's milk (mostly in the United States and Great Britain) and formulas using protein reduced (hydrolyzed) into its component amino acids for infants who are allergic to other proteins. An upswing in breastfeeding in many countries has been accompanied by a deferment in the average age of introduction of baby foods (including cow's milk), resulting in increased use of both breastfeeding and infant formula between the ages of 3–12 months. Nonetheless, only limited information exists about the presence of PCBs and other POPs in commercial infant food (Loran et al., 2010, Weijs et al., 2006).
In humans a wide variety of health effects have been linked to high exposure to PCBs, including developmental defects, reproductive effects and chloracne, hormonal dysfunctions, reduced mental performance, endometriosis and cancer (Guo et al., 2000). Especially during the last two decades, it has been hypothesized that various human male reproductive disorders, such as testicular germ cell cancer, cryptorchidism, hypospadias and low sperm counts, have a common aetiology and may be related to increased PCBs exposure in utero or in early human life (Sharpe, 2003).
Among the other organochlorine pollutants, the authors analyzed residual quantities of PCBs in human milk samples in the Province of Vojvodina. This kind of research was initiated by the same group of authors more than a quarter of a century ago (Vukavic et al., 1986, 1997, 2003, 2008).

MATERIAL AND METHODS

Donors of colostrums were 22 healthy mothers from the city of Novi Sad and its surrounding. They filled in questionnaire contained data on food habits, age, weight, occupation and exposure to chemicals. Mothers expressed colostrum into specially prepared glass containers. Samples were frozen at -20 °C until analyzed. n- Hexane and concentrated sulfuric acid were added to milk samples and cooled. The hexane layer was separated and cleaned up twice with concentrated sulfuric acid and through the Florisil column, and the eluate evaporated and analysed by GC-ECD (HP 5890 supplied with a Quadrex fused silica column 5% Ph for PCBs).
In addition, five infant formulas were analyzed in 2006. in order to compare concentrations of POPs with the concentrations in human milk samples. Infant formulas were purchased in retail by the method of random choice. One was domestic and four were imported infant formulas. Results were statistically processed using IBM SPSS Statistics 15.0 (as $p<0.01$ the correlations were significant).

RESULTS AND DISCUSSION

The age of mothers, donors of colostrum on the 3rd day after delivery, as well as their weight before and after pregnancy were presented in Table 1. Nine of them were primiparous and thirteen multiparous. Mean birth weight of infants was 3351.8 g with mean height of 49.6 cm (Table 1).

Table 1. General demographic characteristics of mothers and infants, presented as mean standard deviation (SD) and range (min-max)

	Mean	SD	Range
Mothers			
Age (years)	28.73	2.45	25-32
Weight before pregnancy (kg)	61.5	10.2	51-87
Weight after pregnancy (kg)	77.9	10.9	67-102
Infants			
Birth weight (g)	3351.8	313.5	2930-3910
Birth height (cm)	49.6	1.7	47-53

Concentrations of 8 EPA PCB congeners were analysed in 22 human milk samples and in five infant formulas (one domestic and four imported). Six PCB congeners were detected in human milk samples, and four were detected in infant formulas. PCB 118 and PCB 28 were not found in any of analyzed samples. The highly chlorinated PCB 209 was found in only two milk samples. Average concentrations of PCB congeners varied from 0.09 (PCB 209) to 0.56 ng/g ww (PCB 101) in human milk samples. In all five samples of infant formulas concentrations were lower than in human milk samples. In domestic formula (manufactured in Serbia) no PCB congener was found. The most dominated congeners were PCB 153, 138 and 180 as it was found in earlier studies.
Comparison of mean concentrations of PCB congeners in human milk samples (n=22) and infant formulas (n=5) was presented in Figure 1.
Between the PCBs concentrations and age of mothers and birth weight of babies correlation was not found. Correlations between PCB 138, PCB 180 and sum of PCB congeners were significant at the 0.01 level (Table 2).

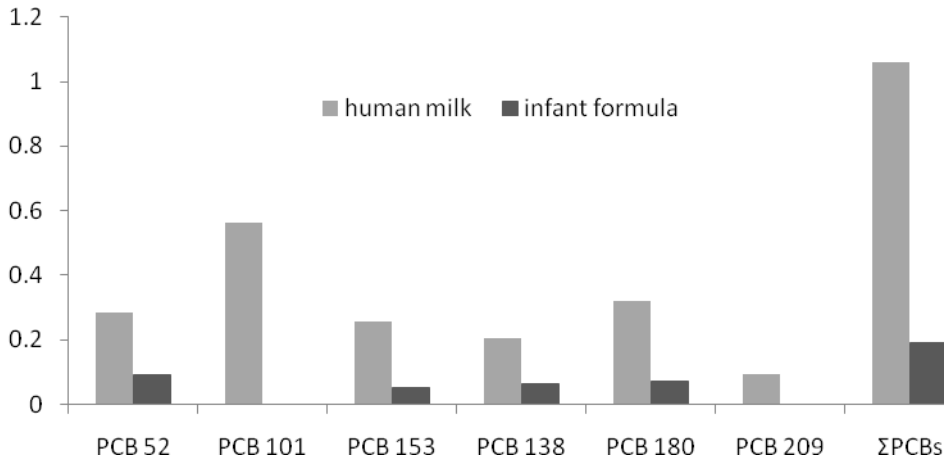

Figure 1. Average concentrations of PCBs (ng/g whole milk) in human milk (n=22) and infant formulas (n=5)

Table 2. Pearson correlations between PCB congeners, age of mothers and birth weight of babies

	PCB52	PCB153	PCB138	PCB180	∑PCBs	b.w.	a.m.
PCB52	1.000	0.508	0.442	0.476	0.561	0.118	-0.001
PCB153		1.000	0.989 (**)	0.992 (**)	0.996 (**)	0.009	-0.038
PCB138			1.000	0.987 (**)	0.984 (**)	0.043	-0.010
PCB180				1.000	0.989 (**)	-0.029	-0.028
∑PCBs					1.000	0.049	-0.024
b.w.						1.000	0.508
a.m.							1.000

*** the correlation is significant at the 0.01 level*
b.w. –birth weight of babies
a.m. – ages of mothers

CONCLUSIONS

22 samples of human milk from healthy mothers were analyzed for the presence of 8 PCB congeners and results were compared with results obtained for 5 analyzed infant milk formulas. No correlation was found between age of mothers and PCB congener levels. Higher exposure of babies to PCBs was estimated for human milk samples than for infant formulas of artificial milk. However, breastfeeding should be encouraged since human milk presents the indispensable food for infant's development and growth. This research of PCBs concentration levels in Serbia is very sensitive and scarce, and till now, performed only by this group of authors.

ACKNOWLEDGEMENTS

This study was funded by Project No. III46009 of Ministry of Education and Science, Republic of Serbia and Project No. 114-451-00603 of Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina.

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RELATIONSHIP BETWEEN WINTER WHEAT QUALITY PARAMETERS AND G × E INTERACTION

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ABSTRACT: Development of new production technologies and quality improvement of traditional wheat products increase the competitiveness and food safety of these products both on domestic and international markets. Growers and users may differ widely in their estimates of wheat quality. Development of new genotypes, highly adapted to particular agroecological conditions, is crucial for obtaining high-quality end products. The aim of this study was to analyze the genotype-environment interactions (GEI) occurring under different agroecological conditions and to estimate their effects on two wheat quality parameters, farinograph quality number (FQN) and baking score (BS). These parameters serve as indicators of dough softening degree and elasticity and crumb structure in bread considered as the end product. The average values of FQN ranged from 43.6 to 72.1, the values of BS from 2.8 to 5.5. The analysis of variance of the genotypes grown in 15 environments showed that the effects of genotype, environment and GEI were highly significant for both analyzed traits. Stability of these parameters in 20 wheat genotypes differing in high molecular glutenin subunits (HMW GS) was studied in five locations for three years. The genotypes with a high FQN were more stable than the genotypes with a high average value of BS. The genotypes with different HMW GS values showed no significant difference in average values for FQN. Significant differences for BS, however, indicated that the final quality is affected by many factors. All these factors should be carefully analyzed in order to provide consumers with top quality products, which is a basic condition for their long-term positioning on the market.
Key words: w*heat, farinograph quality number, baking score, G × E interaction*

INTRODUCTION

Wheat, a major foodstuff and raw material for the milling and baking industries, takes an important place in the economies of many countries. Considering its complex nutritional value, it is important to produce the best possible raw material for human consumption. However, wheat quality is a debatable point, which depends on whether it is viewed from the aspect of growers or users, but in any case it calls for continual improvement (Hristov et al., 2010a).

Development of new production technologies and quality improvement of traditional wheat products are important for the competitiveness and food safety of these products both on domestic and international markets. Quality of wheat-based products is the key factor which secures their position on the market. In other words, it calls for continual development of high-yielding wheat varieties as a way of creating a brand name of high quality raw materials for the milling and baking industries (Kosanović, 2007).

Development of new genotypes, highly adapted to particular agroecological conditions, is crucial for obtaining high-quality end products (Hristov at al., 2010b). Success in breeding depends not only on the genotype but also on environmental factors. Successful wheat breeding of is based on the knowledge of characteristics of wheat genotypes, as well as their interactions with the environment (Zečević at al., 2009). Understanding these relationships may serve to determine the breeding strategy, to identify favorable conditions for testing, as well as to zone the new genotypes for the different agroecological conditions.

The aim of this study was to analyze the effect of the genotype-environment interaction (GEI) on two wheat quality parameters, farinograph quality number and baking score.

MATERIAL AND METHODS

Twenty winter wheat genotypes (G): Pobeda; NSR 5, Mina, Milica, Jarebica, Pesma, Renesansa, Tera, Nevesinjka, Evropa 90, Kremna, Prima, Zlatka, Prva, Tiha, Takovčanka, Toplica, Gruža, Bistrica and KG 100, which differed in the high molecular weight glutenin subunits (HMW GS), were studied at five locations (L): Novi Sad (1), Inđija (2), Sremska Mitrovica (3), Kragujevac (4) i Žitorađa (5), for three years (Y): from 2007 to 2009.
The experiment was set up in a randomized block design with three replications. Conventional agricultural practices used in the commercial production of wheat were applied in the experiment.
Two wheat quality parameters were analyzed: farinograph quality number (FQN) and bread crumb structure - baking score (BS). These parameters serve as indicators of dough softening degree and elasticity and crumb structure of bread considered as the end product. FQN shows the stability of dough consistency and it was determined by the ICC 115/1 method (ICC, 1995). BS (on the scale 0–7, where 7 is excellent) is a numerical expression for the organoleptic assessment of bread crumb structure, representing the sum of points for elasticity and crumb structure (Internal standards, 1976).
The analysis of variance for the three-factorial experiment (factors: genotype, location, year) and the AMMI analysis were calculated by GLM and IML procedures of SAS statistical program (SAS 1998).
Stability of the analyzed quality parameters was expressed as the coefficient of regression (b) (Eberhart and Russel, 1966).

RESULTS AND DISCUSSION

The analysis of variance showed that the effects of genotype, environment and GEI were highly significant for both quality parameters. The analysis of variance of the environment indicated that there existed highly significant effects of location and GEI, while the effect of the year was not significant. Dual interaction of the genotypes, with years and locations, showed no significance. In the triple interaction between the genotype, year and location, highly significant effects were noted on both parameters, with the location covering the largest part of the total variance (Table 1). High divergence of selected locations significantly affects the expression of quality indicators (Williams et al., 2008), accelerating the breeding process by shortening the test period and making it easier to spot differences between genotypes (Weikai and Hunt, 2001).
Genotypes with high FQN have gluten of superior quality, which affects the stability and causes a slower softening of dough (Erekula et al., 2009).
The genotype Kremna exhibited the highest FQN value (72.1), the genotype Takovčanka the lowest (43.6). The genotype Tera had the most stable reaction for this parameter (b = 1.01) (Table 2). According to the regression coefficient, genotypes are more stable if the deviation from b=1 is low. According to the LSD test, as many as 11 genotypes with high stability ($0.74 \leq b \geq 1.26$) showed no significant difference. The genotypes with high mean values (above the average for the study) exhibited different responses in terms of stability, from adequate adaptation to adverse environmental conditions (Milica), exceptional stability (Jarebica, Pesma, Tera, Prima), to better response to favorable environmental conditions (Kremna) (Table 2).This was an indication that each genotype had a specific adaptability to the ecological conditions in which it was grown (Mladenov et al., 2001; Marjanović et al., 2011), and that harmonious alignment between the various parameters is of great importance for high quality wheat varieties (Hristov et al., 2010b).

Table 1. Analysis of variance (ANOVA) for means of squares of 20 wheat cultivars

Source of variation	df	FQN	BS
Genotype (G)	19	621.18**	8.39**
Environment (E)	14	2082.26**	13.61**
Year (Y)	2	122.58	1.08
Location (L)	4	2516.55**	7.47**
YL	8	2355.04**	19.82**
GEI	266	64.62**	0.74**
(1) Three-factor ANOVA			
GY	38	52.76	0.74
GL	76	79.91	0.81
GYL	152	59.94**	0.71**
(2) AMMI analysis			
PC1	32	452.38**	4.56**
PC2	30	387.84	3.41
Residual	204	124.78**	1.68**
Replications	2	1415.29	25.74
Error	570	7.54	0.32

**, **, Significant at P=0.05 and P=0.01, respectively*

FQN - farinograph quality number, BS - baking score.

Regarding the BS value, the genotype Milica showed the highest and the genotype Prva the lowest average value, 5.5 and 2.8, respectively. Tronsmo et al. (2003) reported that in addition to environmental factors, protein quality significantly affects the texture and crumb structure in bread, which is directly correlated with BS. Pore structure also depends on rheological characteristics of dough, with flour quality affecting the intensity of intermolecular interactions (Rozyło et al., 2011) and thus the ratio of the small pores in bread. The genotypes Jarebica and Renesansa were most stable (b =0.98). However, except for the genotypes KG-100 and Kremna, the stability of the other genotypes was in the range $0.62 \leq b \geq 1{:}38$, with nonsignificant differences at the significance level of $P < 0.05$. The genotypes with high mean values (above the average for the study), were generally less responsive to changes in environmental conditions, which confirmed that high values of individual properties can be achieved in stable genotypes (Mut et al., 2010). The above-mentioned genotypes KG-100 and Kremna were exceptions, which were considerably responsive to favorable environmental conditions (Table 2).

Eleven genotypes with HMW GS 5+10 had a lower FQN value (59.4) than the general average (59.6), while eight genotypes with HMW GS 2+12 had a higher FQN value (60.4). However, there were no significant differences between the groups with different HMW GSs, which is consistent with the results of Hristov et al. (2010b). Regarding the BS value, the analysis showed an opposite reaction of the genotypes. The genotypes with HMW GS 5+10 had larger (4.6) and the genotypes with HMW GS 2+12 lower average value (3.9). Also, the analyzed groups differed significantly regarding this parameter (Table 2, Figure 1). It is well known that genotypes with HMW GS 5 +10 generally exhibit better quality compared with genotypes with HMW GS 2+12 (Tahir et al., 2011), with a possibility of deviations occurring in some genotypes (Saint Pierre et al., 2008). In this study, a single genotype (Tiha), with HMW GS 2 +10 at Glu-1D locus, was found to have the values of both parameters lower than the average. Similar situations were also observed in genotypes with HMW GS 5+10 (Nevesinjka, Takovčanka, Toplica) and those with HMW GS 2+12 (KG 100, Evropa 90, Prva), with only the genotype Takovčanka exhibiting a significant difference from the general average (Table 2).

Table 2. Mean values, regression coefficient (b) and HMW GS at Glu-1D for the analyzed genotypes

Genotype	FQN		BS		HMW GS at Glu-1D
	Mean	b	Mean	b	
Pobeda	66.5	0.60	5.4	0.85	5+10
NSR-5	61.8	1.34	4.9	1.07	5+10
Mina	63.3	0.78	4.0	0.68	5+10
Milica	66.1	0.19	5.5	0.72	5+10
Jarebica	61.0	0.98	3.8	0.98	5+10
Pesma	62.3	0.96	5.3	1.06	5+10
Renesansa	58.5	0.81	4.8	0.98	5+10
Tera	57.1	1.01	4.9	1.05	5+10
Nevesinjka	58.5	1.26	4.1	1.27	5+10
Takovčanka	43.6	0.67	3.5	1.04	5+10
Toplica	54.6	1.53	3.9	1.23	5+10
Gruža	59.8	1.12	4.1	0.82	2+12
Bistrica	58.0	1.61	4.5	1.31	2+12
KG 100	49.4	0.43	4.0	1.50	2+12
Evropa 90	57.4	0.91	3.5	0.65	2+12
Kremna	72.1	1.46	4.1	1.44	2+12
Prima	69.2	1.02	4.0	0.96	2+12
Zlatka	59.8	1.10	4.5	0.73	2+12
Prva	57.7	1.33	2.8	0.97	2+12
Tiha	55.5	0.86	3.2	0.65	2+10
Average	59.6		4.2		
LSD (0.05)	5.8	0.52	0.6	0.77	

FQN - farinograph quality number; BS - baking score; HMW GS - High molecular weight glutenin subunits.

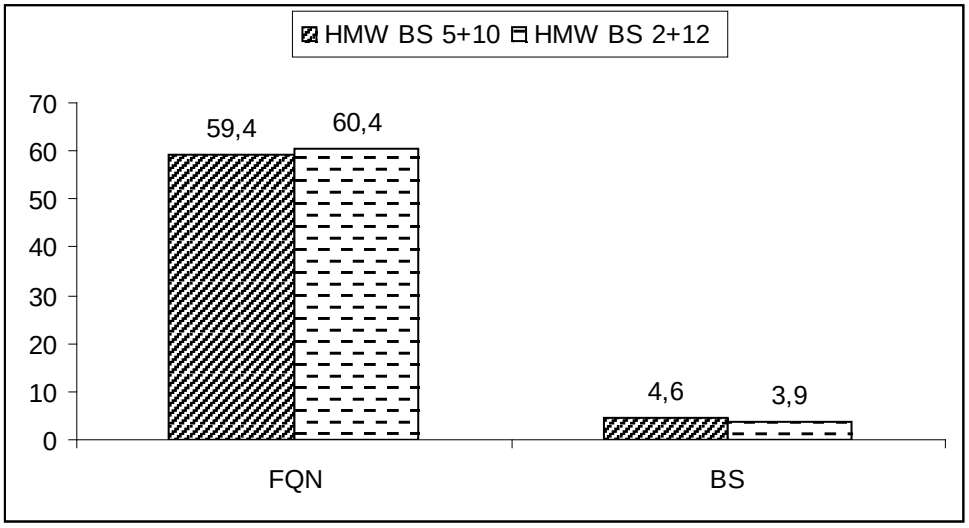

Figure 1. FQN and BS mean values for genotypes with different HMW GS

The year 2008 was most favorable for both parameters under study. On the other side, 2007 was the least favorable for FQN and 2009 for BS. Highest average values for FQN, in all three years, were achieved in the location ŽR. Regarding BS, the highest value was obtained in the location NS. The highest and the lowest FQN stability were established in 2008 in the location SM and in 2008 in the location KG, respectively. The stability of the genotypes studied in these two locations varied significantly (Table 3). Differences in agroecological conditions among locations significantly affect the reliability of the obtained data, which again directly contributes to a shorter test period.

Regarding BS, highest stability was expressed in different years and in different locations (2007 in KG and 2008 in IN). Lowest stability was exhibited in 2009 in the location IN, without significant differences being registered among the observed values of b (Table 3). For

breeders, the stability of quality parameters is important in terms of selection efficiency for individual properties, which directly affects the zoning and growing of certain genotypes in different regions. For milling and baking industries, continual quality of raw materials is crucial for further improvements of the technological process and production efficiency (Mut et al., 2010).

Regression coefficients and the values obtained for BS by AMMI analysis (PC1, PC2) indicated that the studied genotypes reacted similarly to environmental changes. Genotypes with high average values (above the average of the study) were mainly associated with the environments which ensured high BS levels (data not shown).

Table 3. Mean values and regression coefficient (b) for the analyzed environments

	Year	Farinograph quality number					Average
		NS	IN	SM	KG	ŽR	
Mean	2007	59.2	51.3	47.3	61.8	66.0	57.1
	2008	64.8	42.4	53.8	64.7	80.3	61.2
	2009	68.3	53.8	52.6	54.2	73.5	60.5
	Average	64.1	49.2	51.2	60.2	73.3	
b	2007	1.15	1.31	0.77	1.21	1.55	
	2008	1.39	0.70	0.96	1.77	0.66	
	2009	0.60	0.71	0.50	0.80	0.91	
	$LSD_{0.05}$	5.0 (for Mean); 0.71 (for b)					
	Year	Bread score					Average
		NS	IN	SM	KG	ŽR	
Mean	2007	4.6	3.6	3.6	4.8	4.2	4.2
	2008	4.5	2.7	4.0	5.2	5.0	4.3
	2009	5.5	3.3	3.6	3.1	4.9	4.1
	Average	4.9	3.2	3.7	4.4	4.7	
b	2007	1.08	1.58	1.15	1.01	1.12	
	2008	0.92	0.99	0.93	0.81	1.32	
	2009	1.05	0.29	0.61	1.29	0.86	
	$LSD_{0.05}$	0.5 (for Mean); 0.73 (for b)					

NS - Novi Sad, IN – Inđija, SM – Sremska Mitrovica, KG – Kragujevac, ŽR – Žitorađa (locations).

CONCLUSIONS

To ensure a high and stable production of quality raw materials for milling and baking industries, it is necessary to have wheat genotypes that combine superior performance with high technological quality. In addition to genotype, the quality of wheat is affected by production technology and environmental factors. It is crucial to have genotypes capable of maintaining continuity in the expression of quality indicators. It is known that rheological properties of dough affect baking characteristics, which can be taken into account in the breeding process. In this way, processors can get better idea about the different classes of wheat, the different wheat genotypes and the different growing conditions they require and how these parameters affect the mechanism of dough forming and improve the quality of flour and bread.

Since the final product quality is influenced by many factors, it is necessary to carefully analyze all of them in order to offer a steady supply of top quality products to consumers, as a way to secure the position of wheat-based products on the market in the long run.

ACKNOWLEDGEMENTS

The research was funded by Serbian Ministry of Education and Science (Project No. 31066).

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EFFECT OF STORAGE PERIOD ON SEDIMENTATION VALUE (ZELENY TEST) IN WHEAT

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ABSTRACT: Specific conclusions on wheat quality can be drawn from sedimentation value (SV) obtained by Zeleny test (mL). A shortcoming of this method is a reduction of SV during postharvest wheat storage. We studied the dynamics of SV reduction in 15 NS wheat cultivars differing in technological quality. A series of experiments was performed to establish changes in SV depending on the length of wheat storage at room temperature in laboratory. Wheat samples, taken at one-month intervals, were tested for SV (Zeleny test) for one year. The average SV ranged from 20 to 40 mL. The samples with high initial SV generally had a high intensity of SV decline, especially during the last three months of storage. Linear regression showed a significant linear decrease in SV of 0.7 mL per month during the one-year storage period.
Key words: *Wheat, storage, sedimentation value, Zeleny test*

INTRODUCTION

Classification of wheat on the basis of quality parameters is important for other users (food processing industry, human consumption, storage). Specific information on wheat quality can be promptly obtained on the basis of protein content and sedimentation value. The sedimentation value according to Zeleny (Zeleny, 1974) describes the degree of sedimentation of flour suspended in a lactic acid solution for a specified time interval and it is considered as a measure of baking quality. The sedimentation value of flour depends on wheat protein composition that is generally correlated with the protein content or grain hardness (Lasztity,1996). A shortcoming of Zeleny test is that the SV decreases during wheat storage (Ephrat and Sinmena.1976). Total protein content of examined wheat varieties remained unchanged during storage, while Zeleny sedimentation value indicate that quality parameter changes during storage could be highly dependent on cereal variety due to varietal differences in total protein content (Strelec et al.2010).
Wheats with a Zeleny volume (mL) between 22 and 30 are acceptable in EC but they require a machinability test. Wheats with a Zeleny volume (mL) of 30 or higher do not require a machinability test.
Zeleny test is used for preliminary quality testing in the early stages of wheat breeding. Qualitative differences between wheat varieties can be established on the basis of sedimentation value, while modified methods that render SV are needed for a more precise estimation of wheat quality (Kruger and Hatcher, 1995). The Zeleny and the sodium dodecyl sulphate (SDS) sedimentation tests (Axford *et al*., 1979) can be used to obtain a semi-quantitative estimation of the amount of glutenin (or indirectly, of general gluten strength).

Differences in the values of SDS and Zeleny sedimentation tests, which describe the quality of gluten proteins according to their ability to swell and settle in a slightly acidic medium, result from different composition and granulation of the tested components (Weegels *et al*. 1996).

MATERIAL AND METHODS

We studied the dynamics of the reduction of SV in samples of 15 NS wheat cultivars grown at Rimski Šančevi experiment field of Institute of Field and Vegetable Crops in Novi Sad in 2009. Each sample consisted of 5 kg of wheat grains taken after harvest. Grain moisture ranged from 12,8% to 13,5%, so that it was not necessary to dry the samples. Samples were kept in metal boxes at room temperature laboratory, so that the moisture remained unchanged. Samples were taken for analyses at one-month intervals for 12 months.
Grain protein content (GPC) and sedimentation value (SV) were monitored during a period of 12 months (from July 2009. to June 2010.).
GPC (N x 5.7 db) of the whole-grain meal was determined according to Kjeldahl, by ICC standard method 105/2 (ICC 1994). Sedimentation value (Zeleny, 1974) was determined according to ICC 116/1 (1994). All determinations were carried out in three replications.
Average values were used for the evaluation of wheat quality. Statistical evaluation involved the data processed by MS Excel. The effect of storage duration on wheat parameters was calculated by the regression equation.

RESULTS AND DISCUSSION

The effect of storage duration on the quality of wheat from the same harvest can be explained by variations in protein content which is correlated with the sedimentation value (Table 1).
The initial average GPC of the varieties analyzed in 2009. was lower (11.9%) than the long-term average GPC (12.5%) in quality NS wheat cultivars. GPC classification is a system that gives buyers and sellers a common language for grouping products in the same way, everywhere in the world.
The lower average protein content is the consequence of specific weather conditions during the period of protein formation (Hristov and MLadenov, 2005; Šeremešić et al., 2008; Đurić and Kobiljski, 2006).
One-year storage of the studied samples caused a slight reduction in the protein content (July 2009. –11.9% on average; June 2010. –11.7% on average). These results are consistent with findings of Mezei et al. (2007).

Table 1. Effect of storage period on sedimentation value and protein content in wheat grains from 2009.

No.	Storage period	Grain protein content (%/dm)	Max. SV (mL)	Min. SV (mL)	Average SV (mL)	(%) SV compared with 100% measured in July 2009.
1	July	11.9	38	20	31.3	100
2	August	12.4	40	20	32.3	103
3	September	11.6	36	24	30.9	98.7
4	October	11.7	34	23	29.8	95.2
5	November	11.6	33	21	28.8	92.0
6	December	11.6	33	21	27.6	88.2
7	January	11.7	30	21	27.4	87.5
8	February	11.5	30	20	26.5	84.6
9	March	11.7	30	21	26.7	85.3
10	April	11.5	30	21	26.2	83.7
11	May	11.5	29	20	24.5	78.3
12	June	11.7	28	20	24.2	77.3
	Average	11.7	28	21	28.0	89.4
	S.D.	0.25			2.62	
	CV (%)	2			9	

PC-protein content; SV-sedimentation value; S.D.–standard deviation; CV-Coefficient of variation

In agreement with GPC, the average initial SV values were lower (31,3 mL) than the long-term average SV (> 35 mL) in quality NS wheat cultivars. Zeleny sedimentation, however, proved to be a useful indicator of the effects of environmental conditions (Anneliese et al. 2005).
Sedimentation values were significantly different (38 mL on average in July 2009, 28 mL on average in June 2010). Average SV varied from max. 40 mL in the second month after storage, to the lowest value of 20 mL which was obtained several times during the analyzed year.
The rate of SV reduction was highest in the last three months, when the average SV were 83.7%, 78.3% and 77.3% compared with 100% measured in July 2009. The cultivars with high starting SV generally showed a greater rate of reduction than the cultivars with low starting values. This was consistent with the results and Ephrat and Sinmena (1976). After 15-month storage of two wheat cultivars, Lukow et al. (1995) found that SV decreased regardless of the temperature in storage boxes, which ranged from -4 °C to 25 °C.
The coefficient of variation (CV) was higher for SV (9%) than for GPC (2%), as well as the values of standard deviation (2.62 and 0.25, respectively). The deviation of wheat quality parameters from initial values was also observed by Brandolini et al. (2010).
Zeleny test values showed a strong variance in time and an evident trend of decline (Figure 1).

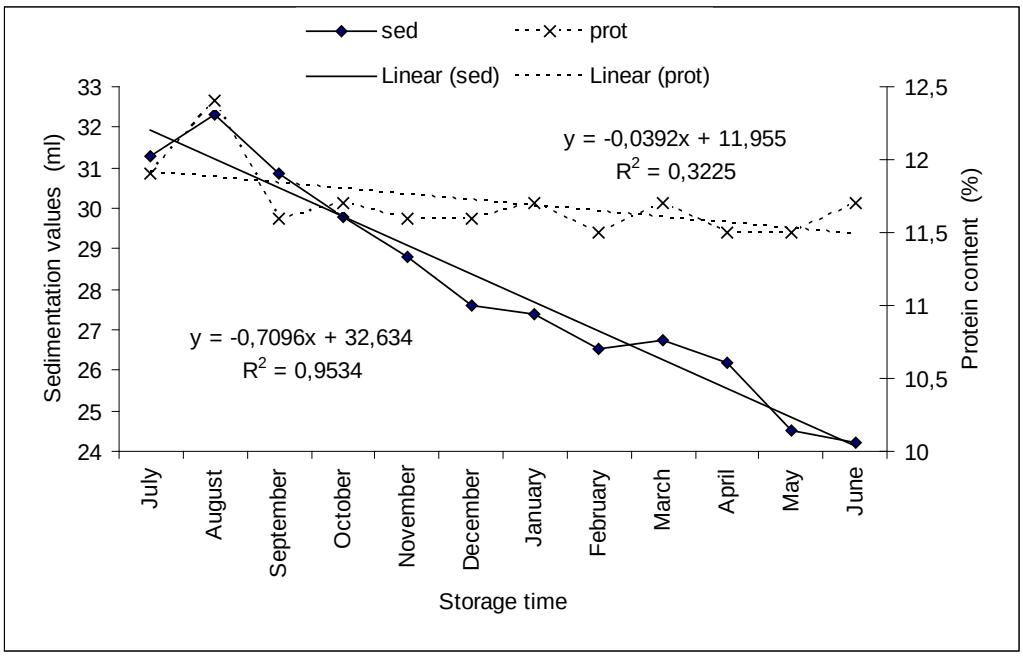

Figure 1. Sedimentation values and protein contents in wheat stored from July 2009. to June 2010. year

We found a slight linear decrease in GPC (0.039%) and a significant linear decrease in SV (0.7 mL per month). The different trends of impact of storage on wheat quality as compared with the results of other authors (Hrušková et al., 2004; Lukow et al., 1995) may be attributed to the production year, choice of cultivar, different conditions and length of storage of wheat samples. Hrušková et al. (2004) found that wheat quality differed from one year to another as well as that wheat quality did not decline inevitably each year.

CONCLUSIONS

After one year of storage of wheat grains under the described conditions, there were no significant changes in total grain protein content.

The vlues of Zeleny test showed a strong variance with time and an evident trend of decline (0.7 mL per month).
Predicting the wheat grain quality based on SV (Zeleny test) was not reliable, because the rate of variation in SV was not consistent among the cultivars which differed in quality and were storaged for different periods of time.

ACKNOWLEDGEMENTS

The research was funded by Serbian Ministry of Education and Science (Project No. 31066).

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ASSOCIATIONS BETWEEN MICROSATELLITE MARKERS AND WHEAT QUALITY PARAMETERS

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ABSTRACT: Wheat quality is a complex collection of traits affected by various loci on multiple chromosomes. This study was conducted in order to identify quantitative trait loci (QTLs) related to most commonly used quality parameters, protein content (PC) and sedimentation value (SED). For this purpose association analysis between SSR molecular markers and quality traits was performed on a selected sample of 94 diverse wheat genotypes. Quality parameters were determined during five years (2007-2011). Microsatellite markers located near targeted QTLs were carefully chosen in accordance with existing literature data. Genomic DNA was extracted from seedlings using CTAB method and PCR products were separated by capillary electrophoresis. The population structure was estimated in Structure v.2.0. software and association analysis was performed in the program Tassel. Significant marker-trait associations (MTAs) were found for the both quality parameters. The markers WMC31 and GWM18 showed significant and stabile associations with QTLs for PC and SED, respectively. Our results demonstrated that association analysis could complement and enhance previous QTL information and provide very useful information for marker-assisted selection.
Key words: bread wheat, protein content, sedimentation, QTLs.

INTRODUCTION

Wheat bread-making quality is highly heritable, controlled by multiple loci and influenced by a number of environmental, genetic and biochemical factors (Zheng et al., 2009). Breeding for improved quality of bread wheat is very difficult and this process can be accelerated by using molecular markers tightly linked to the genes of interest (Bekes et al., 2002; Chen et al., 2009).

Associations between molecular markers and wheat quality characteristics have been studied in two types of populations: structured populations, typically derived from biparental crosses and investigated via quantitative trait locus (QTL) analysis, and non-structured populations, usually collections of cultivars and breeding lines that are analyzed by association analysis methods (Zheng et al., 2009). Non-structured populations have several advantages, including the ability for simultaneous evaluation of different traits using the same genotypic data, more efficient use of resources, better genome coverage and use of multi-location and multi-year phenotypic data at no additional costs, because these data are collected routinely in many wheat-breeding programs (Breseghello and Sorrells, 2006). Due to these advantages, the largest number of marker-trait associations have been established in collections of wheat cultivars and advanced lines (He et al., 2005; Bekes et al., 2006; Chen et al., 2009; Bordes et al., 2011).

This study was conducted in order to identify QTLs related to wheat quality characteristics (protein content and sedimentation value) in a collection of 94 wheat varieties using association analysis.

MATERIAL AND METHODS

Determination of quality parameters

As the material for this study, 94 wheat cultivars were grown in a field conditions during five years (2007-2011). Conventional cultural practices for field experiments were applied as described in Hristov et al. (2010). In the material protein content (PC) was determined by Kjeldahl method ICC 105/2 (ICC 1994), while sedimentation value (SED) was obtained by Zeleny method ICC 116/1 (ICC 1994).

Molecular evaluation

A set of 94 genotypes was fingerprinted using four public available microsatellite markers (WMC31, WMC89, WMC420 and GWM18). Genomic DNA was extracted from approximately 10 young leaves for each variety using modified CTAB protocol (Doyle and Doyle, 1990). PCR amplification was done according to Röder et al. (1998) using fluorescently labeled primers. PCR products were denaturized with HI-DI formamide and fragment analysis was performed on ABI Genetic Analyzer 3130.

Data analysis

Phenotypic data were analyzed using STATISTICA 7 program. Molecular data analysis was performed using Microsoft Excel-Software, and the polymorphism information content (PIC) was calculated according to Anderson et al. (1993). Population structure was determined in software Structure (Pritchard et al., 2000). Q matrix obtained in this program was used for detection of marker-trait associations in Tassel1 (Bradbury et al., 2007). Markers with significant ($P<0.05$) association in the final general linera model (GLM) were declared as main effect QTLs.

RESULTS AND DISCUSSION

Quality parameters

The results have shown that protein content and sedimentation values significantly varied among the genotypes (Table 1). The basic statistic indicators have shown that SED is more variabile trait then PC.

Table 1. Descriptive statistics for protein content (PC) and sedimentation value (SED) in 94 wheat genotypes analysed in five years

Trait	Mean	Confidence -95.0%	Confidence +95.0%	Min	Max	Std. Dev.	Std. Error
PC	13.0	12.90	13.16	9.5	18.9	1.41	0.0648
SED	40.5	39.7	41.28	20.0	70.0	8.57	0.3938

Molecular evaluation

In addition to observed phenotypic variability, molecular evaluation also has shown a considerable level of genetic variability in the material. A total of 28 alleles were detected in the present study at four wheat microsatellite loci, with average allele number per marker of 7 (Table 2). The number of alleles for the individual markers ranged from five (*Xgwm513*) to nine (*Xgwm437*). In the three out of four microsatellite loci null alleles were detected. Polymorphism information content (PIC) ranged from 0.19 for WMC420 to 0.72 for GWM18. An average PIC value of 0.57 for all markers indicated the high level of detected polymorphism.

A similar number of allelic forms with the relatively high level of polymorphism per locus were also detected in other studies (Kobiljski et al., 2002, Maccaferri et al., 2008, Brbaklić et al., 2010), indicating a presence of significant genetic variability in tested materials.

Table 2. Number of detected alleles, product size and PIC-values of four microsatellite markers

Marker	No. of alleles	Product size	PIC
WMC31	6	126-138	0.71
WMC89	9	null, 122-138	0.68
WMC420	5	null, 118-124	0.19
GWM18	8	null, 178-196	0.72
Average	7		0.57

Association analysis

Prior the association analysis, the population structure of chosen genotypes was determined using the program Structure. The genotypes were clustered into three subpopulations according to admixture model used by software. After correction proposed by Evanno et al. (2005) the final number of subpopulations was two. An important prerequisite for association analysis is an existence of certain level of population structure in order to eliminate false positive associations (Prichard et al., 2000; Cockram et al., 2008).

The results of significant marker-trait associations in five analyzed years are shown in Table 3. The marker GWM18 showed significant (P<0.05%) associations with SED in three analyzed years and with PC in the year 2010. The marker WMC31 was significantly associated with PC in three out of five analysed years. The QTL located near to marker GWM18 explained from 15.2 to 26% of total variability for SED, while the QTL near marker WMC31 explained from 13.5 to 22.4% of total variability for PC.

Table 3. Associations between microsatelite markers and wheat quality prameters

Marker	Years	% Proteins		Sedimentation	
		P value	R^2 (%)	P value	R^2 (%)
GWM18	2007	0.7223	4.1	**2.35E+00****	**26**
	2008	0.9997	0.3	0,752	4
	2009	0.3001	8	**0.011***	**15.2**
	2010	**7.60E+00****	**23.6**	**0.0033****	**18.1**
	2011	0.5193	5.7	0.2121	8.4
WMC31	2007	0.096	9.1	0.8683	1.5
	2008	0.0625	10.4	0.3459	5.4
	2009	**0.0011****	**20**	0.5774	3.1
	2010	**3.89E+00****	**22.4**	0.1264	7.7
	2011	**0.0167***	**13.5**	0.819	1.7

**Significant at P<0.05*
***Significant at P<0.01*

Many QTLs for quality traits were identified in different regions of individual wheat chromosomes (Bekes et al., 2002; Chen et al., 2009; Zheng et al., 2009). In the present study, the detected QTLs for SED and PC were found in the regions on chromosome 1B near markers GWM18 and WMC31, respectively. These results are partly in agreement with the results of Wu et al. (2008). They detected three QTLs for kernel protein content on chromosomes 3A and 3B and three QTLs for Zeleny sedimentation value on chromosomes 1B, 1D and 3B. The QTLs on chromosome 1B and 1D were stabile across three environments, explaining 5.5%-17.6% of phenotypic variability. The QTLs controlling Zeleny

sedimentation value, and some other quality traits such as mixing time, eight minute width, peak viscosity and breakdown were mapped on the same region of chromosome 1B, with genetic distances of 0.1-0.8 cM from the nearest marker Glu-B3j, indicating the big influences of 1BL/1RS translocation on these traits. In addition, on chromosome 1D, one QTL was found to control the Zeleny sedimentation value, mixing time and eight minute width, with genetic distances of 2.5-3.3 cM from the nearest marker Dx5+Dy10, exhibiting a great effect of HMW glutenin subunits Dx5+Dy10 on these traits (Wu et al., 2008).
Macaferri et al. (2008) indicated that only stable QTLs expression in a wider range of agro meteorological conditions is one of the determining factors in successful application of candidate markers in MAS for high yield, quality and adaptability in wheat. The both QTLs detected in our study were significant in three of the five tested years, indicating significant environmental effect on these traits. Similar results were found in the study of Kumar et al. (2007), suggesting that the major part of genetic variation for grain protein content is due to environmental interactions. The results of Mann et al. (2009) also suggested the inconsistent genetic control of protein content across the test sites, with only two loci (3A and 7A) showing QTL at three of the five sites.

CONCLUSIONS

The results of the present study have shown significant phenotypic and molecular variability in the population of 94 wheat varieties, which makes it suitable for the detection of important QTLs. Significant associations were found between marker GWM18 and sedimentation value, and between marker WMC31 and protein content. The obtained marker-trait associations should be validated in other material and in more environments in order to check their applicability in MAS.

ACKNOWLEDGEMENT

The research is a part of the project 031066:"Modern breeding of small grains for current and future needs", funded by Ministry of Education and Science, Republic of Serbia.

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CHEMICAL CHANGES DURING THE MANUFACTURE OF BEVERAGES MADE FROM MILK, WHEY AND SOYMILK USING KEFIR STARTER CULTURE

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ABSTRACT: Kefir is a traditional popular middle eastern drink originates from the Caucasus in central Asia. In this study kefir starter culture were used for production of beverages made from milk, whey and soymilk. The incubation time (24 h) and temperature (25 ºC) inoculation rate (5%) and mixing rate (90 rpm) were the same for the all products. Some chemical characteristics (carbohydrate, ethanol, dry matter, fat and pH value) were determined during 48 h of fermentation in 3 beverages. During the fermentation carbohydrate content (w/w) and pH decreased markedly, but dry matter and fat content of the beverages were not significantly different from dry matter and fat content of the substrates. Ethanol content in 3 beverages increased significantly during the fermentation.
Key words: *kefir starter culture, soymilk, fermented beverage*

INTRODUCTION

Kefir is a fermented milk product that has its origin in the Caucasian mountains, Tibet or Mongolia, many centuries ago (Cais-Sokolinska et al., 2008). Soymilk is a liquid extract from soaked and ground soybeans (Smith et al., 2009) which is widely recognized for its high nutritional value owing to its quality proteins. In addition, it is also recognized to contain isoflavones, the bioactive compound that has been extensively associated with the prevention of chronic diseases such as atherosclerosis, cancer, osteoporosis and menopausal disorders (Ewe et al., 2012).

The nutritive quality of soy bean protein is at the pinnacle of food sources available from the plant world. When soymilk compared with cow's milk, the high amounts of arginine and aspartate should be mentioned (Lee et al., 1990) .The polyunsaturated/saturated ratio of soymilk fatty acids is also high (1/1:0.3) along with its concentration of folate (Holland et al., 1991).

Many consumers find the taste of plain soymilk unappealing (Liu and Line, 2000). The distinct aroma of soymilk may be one factor for the low consumption of soymilk in western diets. The state of soymilk can be improved by decreasing the beany, grassy or soy flavor by lactic acid bacteria fermentation (Mital et al., 1974; Granata and Morr, 1996; Liu and Line, 2000).

Milk whey is a by-product of the cheese-making industry which presents about 85-95% of the milk volume and contains nutrients such as lactose, soluble proteins, lipids, minerals, vitamins and organic acids. Because of its high organic matter contents, milk whey can be a serious environmental problem with lactose being mainly responsible for its high BOD and COD values (Mawson, 1994).

Whey is the liquid effluent of the dairy industries and therefore it is produced in large capacities worldwide, creating serious environmental pollution problems (Koutinas et al., 2007). The dairy industry must therefore try to attain a position where handling the whey does not prevent the industry from meeting the market demand for its products. Using the whey for

the production of fermented beverage may reduce its pollution potential while results in the production of a value added product (Ben-Hassan et al., 1995).
In previous studies of Motaghi et al. (1997), the microorganisms from kefir grain were isolated and purified. In the present investigation, the starter cultures were prepared from native microbial strains. Various ratios of starter culture were made from the isolated microorganisms of kefir grain. Lactic acid bacteria, yeast and acetic acid bacteria were used for production of beverage and the products were examined and analyzed for protein, fat, sugar, alcohol, carbon dioxide, acidity, density and riboflavin content.
The aim of this study was to evaluate chemical changes during the fermentation of beverages made from milk, whey and soymilk using kefir starter cultures and mixed cultures consisting of certain species of kefir grain microorganisms in order to develop a procedure where lactic acid fermentation is followed by yeast fermentation.

MATERIAL AND METHODS

Stock cultures

Seven species of bacteria previously identified as *Lactobacillus kefir, L. brevis, L. casei, L. plantarum, Stereptococcus lactis, Leuconostoc mesenteroids and Acetobacter aceti* were combined to form a bacterial starter culture of kefir. Among the isolated yeast from Iranian kefir grain *Candida kefir, Saccharomyces lactis and S. fragilis* were previously isolated and identified by Motaghi et al. (1997). The stock cultures of these organisms were obtained from Persian Type Culture Collection (PTCC). Sterilized skim milk is suitable medium for kefir starter cultures and all cultures were maintained in this liquid.

Microbial enumeration

The preparation of the inoculum of the pure cultures was performed according to the method of Marshall and Cole (1985). Slants were incubated aerobically at 30 ºC for *Streptococci*, for *lactobacilli* under an atmosphere of 80% N_2, 10% H_2 at 30 ºC and *Acetobacter* slant was incubated aerobically at 26 ºC.
The turbidity of each microbial suspension was estimated by spectrophotometer at 550 nm according to the method of Kingra and Horikoshi (1988). Starter bacterial and yeast cultures were prepared in such a way that their concentration (cells/mL) was exactly equal to what they were in one gram of original kefir grain (Motaghi et al. (1997).

Beverage preparations

In the present study, preparation and fermentation of soymilk were performed according to the procedures described by Wang et al. (2002).Whole soybeans with Sahar variety were first washed and soaked overnight in distilled water, after decanting the water, the soaked soybeans were mixed with 10 times the weight of distilled water and comminuted in blender (National MJ-176NR model) for 3 min. The resultant slurry was then filtered through a double layered cheese cloth to yield soymilk. Soymilk was dispensed into containers and autoclaved for 15 min at 121 ºC. Soymilk was used as a growth medium with the following approximate composition: proteins 3.0%, lipids 2.5%, sugars 3.5%, ash 0.5% and water 90.5%. The sugar fraction comprised 2.8% sucrose and 0.7% stachyose. Prior to further use Soymilk was sterilized at 115 ºC.
Milk was homogenized and pasteurized at 85 ºC for 25 min and cheese whey was provided by Kalber S.A. which is a factory processing milk for cheese and other dairy products in the area of Arak that is situated in center of Iran.
The pasteurization technique for whey included heating to 60 ºC for 30 min, follow by cooling to 0 ºC for 30 min and letting stand at room temperature heating. Cooling and standing at room temperature was repeated three times to destroy any vegetative or spore cells present in the whey.
The plate count test (Messer et al., 1985) was performed to ensure the effectiveness of the pasteurization technique. The incubation time (24 h), temperature (25 ºC) and shaking speed

(90 rev/min) were kept constant. The procedures for making various beverages are as follows:
Beverage 1 (B_1): The pasteurized milk was inoculated with combined kefir grain bacteria with yeast inoculate was used (3% (v/v) bacterial strain and 2% (v/v) yeast kefir grains previously described by Mazaheri et al. (2000).
Beverage 2 (B_2): The pasteurized whey was inoculated with combined kefir grain bacteria with yeast inoculate was used (3% (v/v) bacterial strain and 2% (v/v) yeast kefir grains previously described by Mazaheri et al. (2000)
Beverage 3 (B_3): The sterilized soymilk was inoculated with combined kefir grain bacteria with yeast inoculate was used (3% (v/v) bacterial strain and 2% (v/v) yeast kefir grains previously described by Mazaheri et al. (2000).
From each batch of beverage, samples were taken at 0 h and after 24 and 48 h of fermentation (incubation).

Chemical analysis
Fat contents of individual sample were measured according to standard AOAC method 2000 18. AOAC, method 990. 19 was used to determine total solid in each sample. The pH was determined by inserting a pH probe (pH meter Micro pH 2002; Crison, Barcelona, Spain) directly into a well-mixed sample of the sample.
Carbohydrate was measured by HPLC (Waters, Mil ford, MA, USA) equipped with an Interaction ION 300 column (Phenomenex, Torrance, CA, USA), using H_2SO_4 (0.0064 N) as mobile phase at a flow rate of 0.4 ml min $^{-1}$ (2).
Ethanol was determined using a gas chromatograph. GC analysis was carried out on a Shimadzu Auto system gas chromatograph equipped with a flame ionization detector (FID). A CBP_{10} capillary column (L = 25 m, D = 0.33 mm, df = 0.5) was used. Helium was used as a carrier gas. The injector was heated to 100 ºC and the FID to 260 ºC and the column temperature program was isothermal at 90 ºC. The samples were injected in the split less mode (Wang et al., 2004).

Statistical analysis
All results presented in this study are the mean of three independent experiments with three replicates each. Data were analyzed by the general linear model procedures of the statistical analysis system (SAS, 1986); $p<0.05$ was considered significant. Means were compared by last significant difference.

RESULTS AND DISCUSSION

In present study by using the native microbial strains isolated from kefir grains starter culture, the following results were obtained.
Table 1 shows that mean changes in some chemical components during fermentation of milk in the manufacture of beverage 1. Table 2 shows that mean changes in some chemical components during fermentation of whey in the manufacture of beverage 2 and Table 3 shows that mean changes in some chemical components during fermentation of soymilk in the manufacture of beverage 3.

Table 1.Changes in some chemical components (%, w/w) during the 48h of fermentation of heat treated cow's milk.

	Fermentation time (h)		
	0	**24**	**48**
Carbohydrate	4.90 ± 0.22	4.02 ± 0.16	3.96 ± 0.18
Ethanol	0.002 ± 0.002	0.005 ± 0.003	0.008 ± 0.004
Dry matter	11.90 ± 0.52	11.70 ± 0.54	11.40 ± 0.50
Fat	3.71 ± 0.18	3.60 ± 0.12	3.47 ± 0.20
pH	6.30 ± 0.10	4.40 ± 0.20	4.30 ± 0.10

Table 2. Changes in some chemical components (%, w/w) during the 48h of fermentation of heat treated whey

	Fermentation time (h)		
	0	**24**	**48**
Carbohydrate	4.36 ± 0.26	3.89 ± 0.12	3.80 ± 0.28
Ethanol	0.002 ± 0.001	0.004 ± 0.004	0.006 ± 0.003
Dry matter	5.70 ± 0.32	5.60 ± 0.24	5.50 ± 0.40
Fat	0.57 ± 0.08	0.50 ± 0.10	0.41 ± 0.06
pH	5.50 ± 0.20	4.60 ± 0.1	4.50 ± 0.20

Table 3. Changes in some chemical components (%, w/w) during the 48h of fermentation of heat treated soymilk

	Fermentation time (h)		
	0	**24**	**48**
Carbohydrate	3.50 ± 0.18	3.20 ± 0.14	3.10 ± 0.18
Ethanol	0.002 ± 0.001	0.003 ± 0.004	0.004 ± 0.002
Dry matter	9.50 ± 0.44	9.35 ± 0.42	9.24 ± 0.46
Fat	2.50 ± 0.14	2.40 ± 0.10	2.32 ± 0.10
pH	6.70 ± 0.10	5.40 ± 0.10	5.00 ± 0.20

The Carbohydrate content decreased significantly ($P<0.05$) during the first 24 h, and then more slowly until the 48 h of fermentation.

Carbohydrate was consumed during the 24 h fermentation period, and carbohydrate levels decreased by 20–25% with respect to the initial carbohydrate levels present in the milk and whey. In milk substrate, the final carbohydrate contents were within the range of the values observed by other authors for Kefir produced from milk obtained from different species (Muir and Hunter, 1992; Muir, Tamine, and Wszolek, 1999). In soymilk carbohydrate levels decreased slowly. Alm (1982) did not detect galactose in the kefir samples, a finding repeated here in our study. This is because the galactose formed by hydrolysis of the lactose is employed by the kefir microflora to form the polymer kefiran characteristic of kefir, used to make the new granules formed during the fermentation process.

Assadi, Pourahmad, and Moazami (2000) manufactured kefir in the same conditions employed here using a 5% inoculate and reported lactose levels of around 1.4% after 24 h of fermentation, much lower than the levels recorded in the present experiment.

The ethanol content increased slightly, but significantly ($P<0.05$), during the period from 0 to 48 h, reaching a mean final value of 0.008% (w/w); this value is within the range of values observed by other authors for Kefir from different origins (Gu¨ zel-Seydim et al., 2000; Kurman, Rasic, and Kroger, 1992; Marshall and Cole, 1985; Wszolek, Tamine, and Muir, 2001). In whey the ethanol content increased slightly, but significantly ($P<0.05$), during the period from 0 to 48 h, reaching a mean final value of 0.006% (w/w); this value is within the range of values observed by other authors Mazaheri Assadi, Abdolmaleki, and Mokarram, (2008).

Dry matter in the three beverages did not significantly change after 48 h of fermentation .Dry matter values were similar to those recorded for other fermented milks (Gambelli et al., 1999) and to those reported by Ching-Yun and Ching-Wen (1999) for kefir made using a 5% inoculate. These values were not significantly different from the dry matter content of the source substrates. Accordingly, as was the case for the fat content, fermentation did not affect the dry matter content of the source substrate used. Ottogalli, Galli,Resmini, and Volonterio (1973) found that the dry matter in recently manufactured kefir differed according to the geographic origin of the granules, with variations in dry matter of between 9.4% and 11.1% in the milk substrate. The percentage of kefir grains inoculated did not significantly influence the dry matter in the samples.

The fat content of three beverages did not differ significantly ($p > 0.05$) from the fat content of the substrates (milk, whey and soymilk). This finding was consistent with reports by other researchers (Gambelli et al., 1999; Huerta-Gonzalez and Wilbey, 2001; Walstra and Jenness, 1987), who observed that in macro nutritional terms, the nutritional composition of

fermented milks, including the fat content, was the same as that of the source milk. In contrast, Ching-Yun and Ching-Wen (1999) observed the fat content of recently cultured kefir to be lower than that of the milk, a difference possibly ascribable to the lipases produced by the kefir grains during fermentation (Vujicic, Vulic, and Konyves, 1992).
There was a sharp decrease of around 2 pH units during fermentation in three beverages. As already related above, the lactic acid bacterial population declined with time, which is why the kefir did not become more acidic. Collar (1996) found that lactic acid bacteria multiply and produce lactic and acetic acids more slowly in mixture with yeasts than in pure culture (Collar, 1996). The percentage kefir grain inoculate added did significantly ($p < 0.05$) affect the pH values which reported by Irigoyen, Ortigosa, and Torrez (2003), who recorded significant differences during kefir manufacture according to the percentage kefir grain inoculate added.

CONCLUSIONS

In the manufacture of Kefir, by fermentation of heat treated cow's milk, whey and sterilized soymilk using kefir starter culture. During the fermentation carbohydrate content (w/w) and pH decreased markedly, but dry matter and fat content of the beverages were not significantly different from dry matter and fat content of the substrates. Ethanol content in 3 beverages increased significantly during the fermentation.

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THE AMOUNT OF THIOL (SH) GROUPS AS A QUALITY INDICATOR OF THE WHEAT FLOUR PROTEIN COMPLEX

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ABSTRACT: Since the technological quality of flour is directly influenced by protein content and quality, a great attention is paid to determination of its properties. For this purpose, a wide range of chemical, physical and rheological tests are commonly employed. The rheological properties of dough during baking processing and ultimately the quality of final product are influenced by the changes of the thiol/disulphide structure of gluten proteins. The aim of this study was to monitor the changes in the amount of thiol groups as a quality indicator of protein complex of three freshly harvested wheat samples from two localities characterized by different micro-climatic conditions in 2010/2011 production year. By modifying the method of Pérez et al. (2005), the content of thiol groups was spectrophotometrically determined in wet gluten of selected samples (λ = 412nm). Each sample was beforehand tempered at two different temperatures (30° and 37 °C) in order to imitate the flour processing conditions as well as to determine the impact of hydrolytic enzymes present. On the basis of obtained results, it was noticed that each variety manifested different trend in the amount of thiol groups over time when tempering at 30 °C, whilst tempering at 37 °C highlighted the dominant impact of selected localities on the amount of thiol groups.
Key words: *wheat, quality, gluten, thiol groups, micro-climatic conditions*

INTRODUCTION

Gluten proteins have a primary influence on the technological quality of wheat flour. In fact, the technological quality mainly depends on glutenin and gliadin fraction which account for 80% of total flour proteins (Shomer et al., 1998; Wieser, 2007). Both fractions influence rheological properties, with different role for wheat flour functionality. It is considered that glutenin fraction contributes visco-elastic properties of gluten and dough. On the other hand, gliadin fractions, by non-covalent interactions with one another and with glutenin polymers, act as plasticizers of gluten mass (Perez et al., 2005; Manu et al., 2008; Shewry et al., 1997). Therefore, the technological properties of flour depend on the structure of the protein fractions and their interactions as well as protein interactions with other components (Shewry et al., 1997).

Glutenin subunits form both intra- and inter-chain disulfide (-S-S) bonds, while the establishment of intermolecular disulfide bonds is characteristic for gliadins, resulting in smaller molecular weight of these proteins (Perez et al., 2005). During the mixing of dough, gluten network is formed as a direct consequence of the linking process by disulfide inter- and intramolecular bonds between monomeric gliadins and polymeric glutenins. Structure of gluten network is determined by the number of available thiol (-SH) groups, so the number of resulting inter-and intramolecular disulfide bonds influences various properties of gluten (Torbica, 2007).

Since the formation of gluten is related to the transformation of -SH groups to -S-S bonds, a great attention is given to examination of their contents because they are generally considered to be important indicator of rheological properties of dough and baking performance of wheat (Chen and Schofield, 1996; Ou et al., 2004).

Taking into account that in the processing of wheat flour dough, the proteins are undergoing certain biochemical changes that are followed by rearrangement or interchange of SH/SS

bonds, the aim of this study was to monitor the changes in the content of thiol groups as a quality indicator of protein complex of three freshly harvested wheat samples from two localities characterized by different micro-climatic conditions in 2010/2011 production year.

MATERIAL AND METHODS

Wheat samples of two Serbian varieties (Pobeda and Zvezdana) and one French variety (Apache) were collected from two localities in Northern Serbia (locality 1 and locality 2) in 2011.
The content of wheat-bug damaged kernels was determined by standard method (Pravilnik o kvalitetu poljoprivrednih proizvoda koji se skladište u javnom skladištu, 2010). Wheat samples were milled to flours using laboratory mill MLU 202 (Bühler, Switzerland) (flour extraction rate of 60%).
Gluten for determination of free thiol group content was washed according to ICC method 137/1, whilst determination of rheological behaviour of dough was performed by using Mixolab (Chopin Technologies, France) according to ICC method 173.
Each sample of wet gluten before examination was tempered at two different temperatures (30 °C and 37 °C) during 45, 90 and 135 min in order to imitate the flour processing conditions as well as to determine the impact of hydrolytic enzymes present.
Concentration of free thiol groups was examined as previously described by Prasada Rao et al (2002) and Perez et al (2005), with two modifications: after vortexing gluten was centrifuged at 14 500 rpm for 6 min and volume of Ellman's reagent was increased for 25%.

RESULTS AND DISCUSSION

Localities 1 and 2 for this study were selected due to the history of climatic conditions during the last decade, why wheat from locality 1 has been mainly characterized by poor technological quality (low values of extensograph energy), whereas wheat from locality 2 has been characterized by good technological quality (high values of extensograph energy) (http://www.fins.uns.ac.rs/index.php?page=studije-o-kvalitetu&hl=sr).
Figure 1 shows the results of determination of thiol groups of gluten samples previously tempered at 30 ^{o}C. According to obtained results, it is noticeable that the changes in thiol status were affected by the variety. The greatest changes occurred after incubation of gluten for 90 minutes. Variety Apache, regardless of geographic origin, did not show significant changes in the thiol groups' content, while the most dynamic changes were noticed in gluten of Zvezdana variety. Zvezdana manifested clearly visible difference in the thiol groups content depending on the growing locality. Gluten of Zvezdana variety from locality 1 tripled the thiol content compared to the untempered samples, indicating that gluten was hydrolised during the incubation, releasing polypeptides. Gluten of Pobeda variety showed the highest stability with regard to thiol groups content, while in relation to the all investigated samples it could be seen that the gluten sample from the locality 2 had the lowest content of -SH groups.

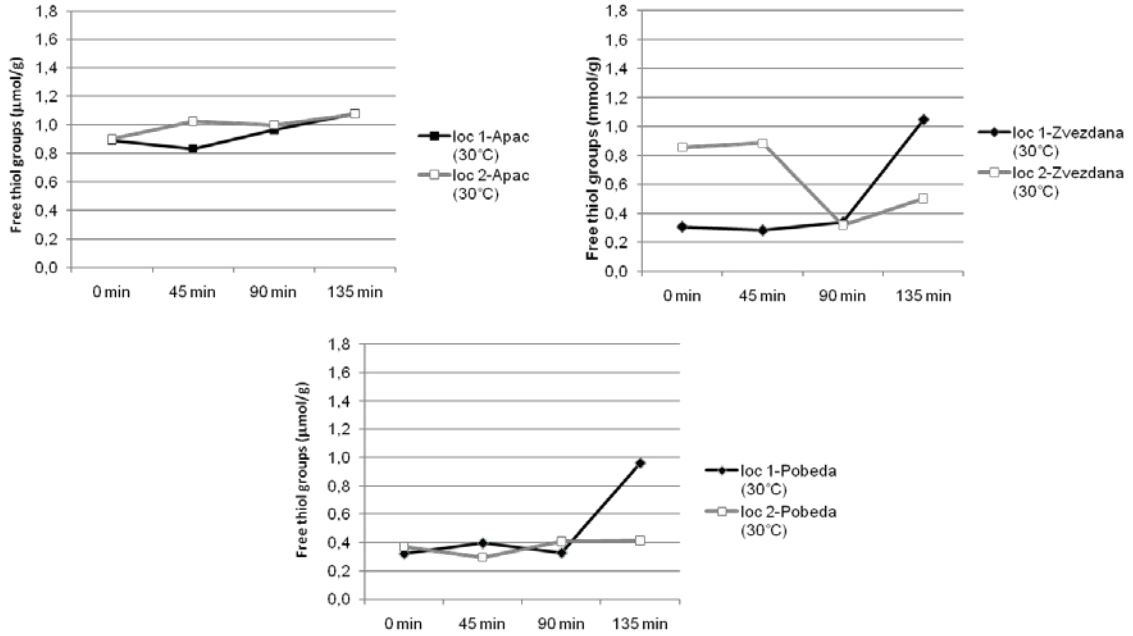

Figure 1. Concentration of free thiol groups of gluten during incubation at 30 ˚C

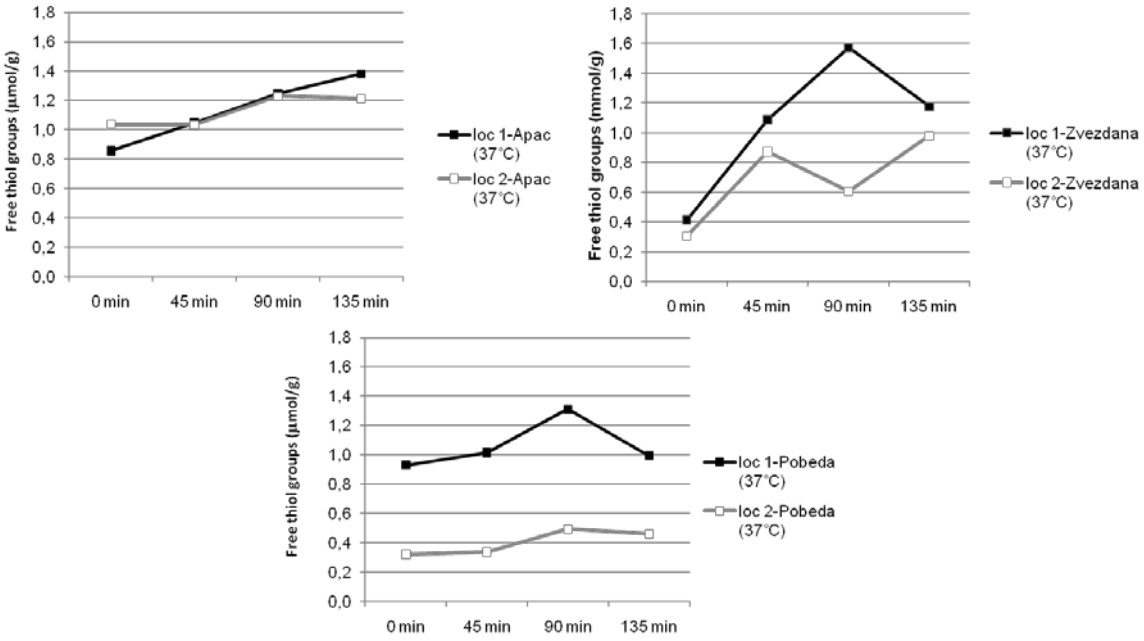

Figure 2. Concentration of free thiol groups of gluten during incubation at 37˚C

By tempering the gluten samples at 37 ^{o}C, different dynamic of changes in the content of thiol groups could be noticed, which could be primarily attributed to the inherent characteristics of the varieties. According to the values of thiol groups content observed from samples previously tempered at 30 ^{o}C, gluten of Zvezdana showed higher values. The changes for other samples were not significantly different. Gluten of Apache variety manifested negligible influence in relation to the locality. The samples of Zvezdana variety were similarly influenced after the incubation time of 45 minutes with an opposite trend in thiol groups content. Gluten samples of Pobeda variety showed nearly identical trend in change, with stronger influence of the locality in comparison to other samples.

Table 1. The selected indicators of technological quality of wheat flour samples

Locality	Sample	W.Abs (%)	Stability time (min)	Amp. (Nm)	Development time (min)	α	C2 (Nm)	Wheat-bug damaged kernels (%)
Locality 1	Pobeda	58.30	9.38	0.08	6.60	-0.026	0.51	0.40
Locality 1	Zvezdana	58.40	10.63	0.06	8.78	-0.086	0.53	0.42
Locality 1	Apache	53.30	10.53	0.09	1.63	-0.110	0.51	1.00
Locality 2	Pobeda	58.50	10.18	0.06	9.00	-0.026	0.51	0.30
Locality 2	Zvezdana	59.30	9.77	0.06	6.80	-0.084	0.49	0.41
Locality 2	Apache	53.30	10.90	0.09	1.40	-0.100	0.55	0.70

The values of selected rheological parameters of investigated wheat flour samples are shown in Table 1. By observing the values of parameters, which are related to the physical characteristics of the protein matrix, it could be seen that the examined samples had similar values of water absorption and dough stability. The dough elasticity, expressed by the value of amplitude (Nm), was slightly higher for flour samples of Apache variety. Dough development time, i.e. time required to reach the proper consistency of the dough, was also shorter for Apache samples, which could be attributed to the genetic origin of the variety. On the other hand, certain rheological parameters (α, C2, Stability time) indicated a better initial protein quality of the Apache samples despite the fact that this variety possessed the highest content of wheat-bug damaged kernel. The value of slope α showed that Apache variety was characterized by the lowest rate of weakening of the protein structure due to the effects of temperature increase and applied forces during dough mixing. Furthermore, it could be noticeable that the resistance of protein structure mainly depends on the characteristics of varieties, because the values of slope α for the observed varieties were very similar regardless the locality.

CONCLUSIONS

Based on the content of thiol groups, content of gluten samples of selected varieties from the two localities previously tempered at 30 °C and 37 °C, it could be concluded that the changes are primarily varietal characteristics. The most significant changes during incubation of gluten at 30 °C occured in 90 minutes. On the basis of selected rheological parameters on the Mixolab (α, C2, Stability time) it can be concluded that the resistance of protein structure mainly depends on the characteristics of variety. In order to achieve more reliable conclusions which can precisely explain how thiol groups influence the quality of wheat flour, further investigations should be conducted in the folowing harvest seasons on the greater numbers of wheat samples from the same locations.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 31007 “Vrednovanje kvaliteta i optimizacija prerade pšenice u svetlu klimatskih promena”, financed by the Ministry of Education and Science, Republic of Serbia.

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INVESTIGATION OF RELATIONS BETWEEN HMW GLUTENIN COMPOSITION AND RHEOLOGICAL PROPETIES OF CONTEMPORARY WHEAT VARIETIES

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ABSTRACT: Among cereal scientists it is widely accepted that rheological properties of dough, examined by different types of equipment, but also the structure of high molecular weight (HMW) glutenin subunits are highly related to wheat baking properties.
Farinograph, Extensigraph and Amilograph properties and lab-on-a-chip (LoaC) electrophoresis analysed composition of HMW glutenins of twenty different wheat varieties grown in three different years (2008, 2009 and 2010) were determined. Based on the occurrence of HMW glutetnin fraction of identified molecular weights (131, 133, 139, 148, 186 and 218 kDa) analyzed wheat varieties were, independently of the growing season divided in four groups under which varieties with the same HMW compositions were comprised. Rheological properties were compared among the groups of varieties with the same HMW glutenin compositions.
The group which differentiated in comparison to the other groups on the basis of presence of the fraction of HMW glutenin with the highest molecular weight of 218 kDa had statistically higher farinograph quality number than other groups. The group in which both HMW glutenin fractions with the lowest molecular weights of 131 and 133 kDa were present was characterized in comparison to other groups with statistically lower farinograph quality number and extensograph energy, and statistically higher farinograph softening degree. In the group which differentiated from the other groups based on presence of HMW fraction of 139 kDa amilograph peak viscosity was statistically higher than in other groups.
Key words*: wheat quality, rheology, HMW glutenin subunnits*

INTRODUCTION

Among cereal scientists it is widely accepted that rheological properties of dough from wheat flour, examined by different types of equipment are highly related to wheat baking properties. Brabender devices such as Farinograph, Extensigraph and Amilograph are well known in the last centuries as the standard methods for flour examinations (ICC 115/1, ICC 114/1 and ICC 126/1, respectively). Nowadays, scientist worldwide still use these techniques for predicting end-use quality for baking industry (Zhang et al., 2007; Hemalatha et al., 2010) and quality of different wheat lines and varieties (León et al, 2009; Rakszegi, et al, 2008).
Also, the structure of high molecular weight (HMW) glutenin subunits is highly related to wheat rheological properties. Hou et. al. (1996) confirmed that positive correlation exists between HMW glutenin subunits content and rheological properties of wheat flour, whereas Antes and Wiesser (2001) emphasize the great influence of HMW glutenin subunits amount in the prediction of the dough or the gluten strength.
During last decade new promising method for investigation of (HMW) glutenin subunits - microfluidic or Lab-on-a-Chip (LoaC) electrophoresis was developed. This technique can be used for fast identification of wheat varieties (Uthayakumaran et al., 2005) and separation, determination and quantitation of HMW glutenin subunits (Rhazi et al., 2009).

Aim of this study is to show how HMW glutenin composition determined by LoaC techinque is related to rheological properties of twenty different contemporary wheat varieties grown in three different years at the same location in Serbia.

MATERIAL AND METHODS

Sixteen different wheat varieties (Renesansa, NS Rana 5, Angelina, Simonida, Pobeda, NS 40S, Bastijana, Rapsodija, Dama, Etida, Arija, Evropa 90, Kantata, Ljiljana, Dragana and Vojvodina) were grown in three different years (2008, 2009 and 2010), whereas three wheat varieties (Nevesinjka, NS3-5299/2 and Zvezdana) were grown in two different years (Nevesinjka in 2008 and 2009; NS3-5299/2 and Zvezdana 2009 and 2010) and Astra wheat variety was grown only in 2008. These wheat varieties, supplied by Institute for field and vegetable crops from Novi Sad, Serbia, were milled by MLU 202 mill Bühler Switzerland to 60% flour yeald. Rheological properties of gained flour of examined wheat varieties were determined by Farinograph, Extensigraph and Amilograph Brabender Germany according to standard Serbian methods (Pravilnik o metodama fizičkih i hemijskih analiza za kontrolu kvaliteta žita, mlinskih i pekarskih proizvoda, testenina i brzo smrznutih testa, Regulation on physical and chemical methods for control of cereals, milling and bakery products, pasta nad fast frozen dough). Composition of HMW glutenin subunits was determined from flour samples (30 mg) which were first extracted once with 300 µl of deionisied water, once with 300 µl 2% salt solution and once with 70% ethanol solution to remove albumin, globulin and gliadin proteins (mixing each time on a vortex mixer for 10 s and centrifuging after 24 h for 20 min at 14500 r/min). The full range of glutenin subunits was then extracted at 100 °C for 5 min with 350 µl of 2% SDS solution containing 5% β-mercapto ethanol. Clarified extract (4 µl) of samples was mixed with 2 µl of Agilent sample buffer and 84 µl of deionisied water and gained mixture of each sample was added to one of the 10 sample wells of the chip. Then the chips with 10 samples were analysed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), in combination with the Protein 230 Plus LabChip kit and the dedicated Protein 230 software assay on 2100 expert software.
The data were statistically treated by analysis of variance (ANOVA) the means were compared by the Duncan test at significance level of 0.05 using the Statistical software StatSoft, Inc. (2011). STATISTICA (data analysis software system), version 10.0 www.statsoft.com.

RESULTS AND DISCUSSION

After separation of glutenin subunits of investigated wheat varieties by LoaC electrophoresis (Figure 1) region of HMW glutenin subunits was identified according to the results Uthayakumaran et al. (2006) who determined that apparent size of HMW glutenin subunits of wheat varieties identified by LoaC electrophoresis is ranged from 128 kDa. In this region six different glutenin subunits with average molecular weigth of 131, 133, 139, 148, 186 and 218 kDa were identified (Figure 1).
Based on the occurrence of certain HMW glutenin subunits, analyzed wheat varieties were, independently of the growing season divided in four groups with the same HMW compositions (Table 1):

- Group A: wheat varieties with HMW glutenin subunits of 131, 133 and 186 kDa,
- Group B: wheat varieties with HMW glutenin subunits of 133, 148 and 186 kDa,
- Group C: wheat varieties with HMW glutenin subunits with apparent size of 131, 139, 148 and 186 kDa and
- Group D: wheat varieties with HMW glutenin subunits with apparent size of 133, 148, 186 and 218 kDa.

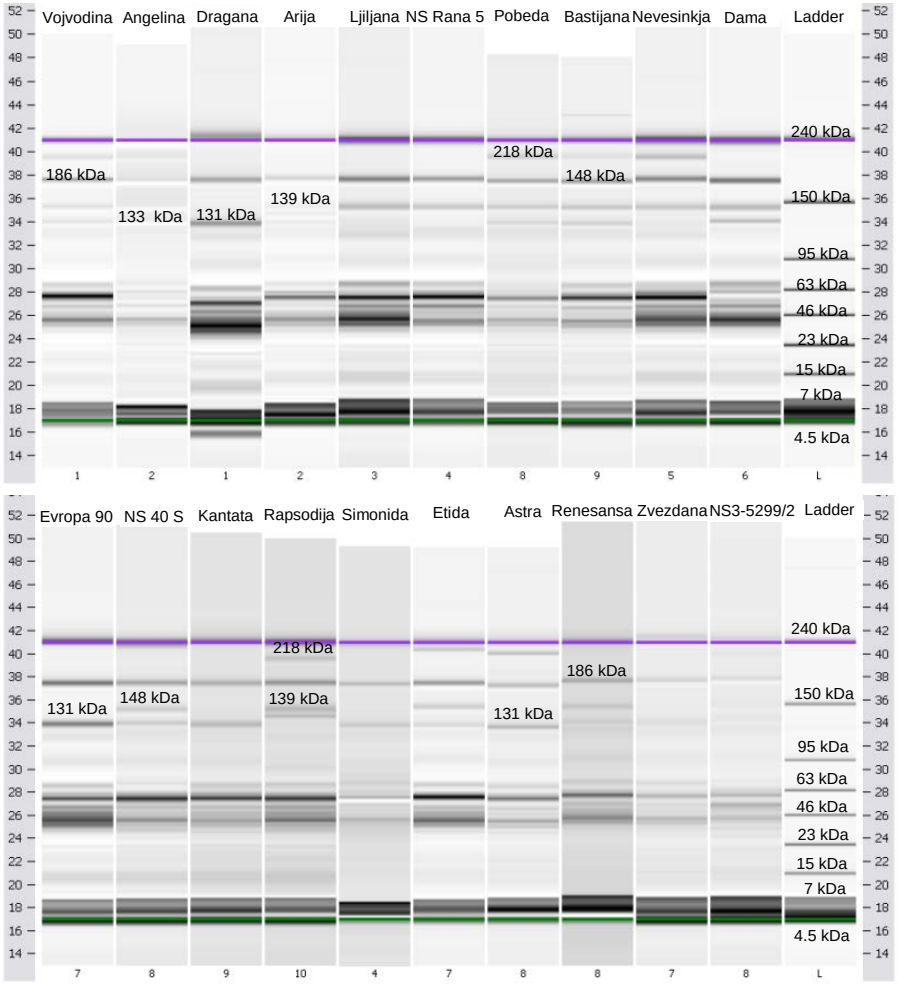

Figure 1. Gel like image of two chips representing glutenin subunits of twenty examined wheat varieties.

Table 1. Groups of wheat varieties with the same HMW compositions

Group A	**Group B**	**Group C**	**Group D**
131, 133 and 186 kDa	**133, 148 and 186 kDa**	**131, 139, 148 and 186 kDa**	**133, 148, 186 and 218 kDa**
Dragana, Evropa 90, Kantata, Simonida, Astra and Zvezdana	Ljiljana, NS Rana 5, Dama, NS 40 S, Etida and Renesansa	Arija, Nevesinjka and Rapsodija	Vojvodina, Pobeda, Angelina, Bastijana and NS3-5299/2

Rheological properties of flours samples of wheat varieties measured by Farinograph, Extensigraph and Amilograph were compared among the groups of varieties with the same HMW glutenin compositions (Table 2). The group D which differentiated in comparison to the other groups on the basis of presence of the fraction of HMW glutenin with the highest molecular weight of 218 kDa had statistically higher farinograph quality number than other groups. Group A in which both HMW glutenin fractions with the lowest molecular weights of 131 and 133 kDa were present was characterized in comparison to other groups with statistically lower farinograph quality number and extensigraph energy, and statistically higher farinograph softening degree, which is in agreement with well known results Payne et al. (1981) that HMW glutenin subunits 2+12 and 4+12 cause lack of dough strength. In the group C which differentiated from the other groups based on the presence of HMW fraction of 139 kDa amilograph peak viscosity was statistically higher than in other groups. However, in the group B was not noticed any statistical differentiation in rheological properties to the other groups of wheat varieties. Also, the groups of wheat varieties did not show statistical

differentiation in several rheological parameters such as water absorption, dough development, dough stability, resistance and extension, which can be explained by the fact that in this study was not included quantification of HMW glutenin subunits.

Table 2. Compared rheological parameters of four wheat varieties groups with the same HMW glutenin composition measured by Farinograph, Extensigraph and Amilograph,

Group	Water absorption (%)	Dough development (min)	Dough stability (min)	Degree of softening (FU)	Quality number	Peak viscosity (AU)	Energy (cm^2)	Resistance (EJ)	Extension (mm)	Resistance/Extension
Group A	61.9^{a}	3.1^{a}	1.7^{a}	88^{ab}	59.0^{a}	590^{a}	64.2^{a}	214^{a}	161^{a}	1.43^{a}
Group B	61.8^{a}	2.6^{a}	2.2^{a}	69^{ab}	64.5^{ab}	430^{a}	89.6^{b}	278^{a}	160^{a}	1.81^{a}
Group C	61.1^{a}	2.5^{a}	2.7^{a}	61^{a}	65.0^{ab}	1055^{b}	88.3^{b}	264^{a}	157^{a}	1.71^{a}
Group D	64.0^{a}	2.9^{a}	3.1^{a}	51^{a}	70.5^{b}	575^{a}	94.7^{b}	266^{a}	166^{a}	1.67^{a}

Mean values of examinated quality parameters calculated on dry matter labeled by the same letter in raw were not statistically significant difference ($p<0.05$).

CONCLUSIONS

It can be concluded that it is confirmed that relation among some rheological parameters such as farinograph degree of softening and quality number, amilographs peak viscosity and extensigraph energy, of flour of wheat varieties with different HMW glutenin structure exists. For better understanding of connections among examined groups of wheat varieties and composition of HMW glutenin subunits, it is probably needed to determine the quantity of determined HMW glutenin subunits.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 31007 “Vrednovanje kvaliteta i optimizacija prerade pšenice u svetlu klimatskih promena”, financed by the Ministry of Education and Science, Republic of Serbia.

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ANALYTICAL METHODS FOR BIOTIN DETERMINATION

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ABSTRACT: This paper gives a rewiew of analytical methods for biotin determination in various mediums. Contemporary analytical methods provide fast biotin determination with low detection limits. Results of quantitative determination of biotin in sugar beet molasses obtained by various methods are presented.The following methods were applied: HPLC (High Pressure Liquid Chromatography), TLC (Thin Layer Chromatography), microbiological method by Danielsen and Eriksen (1968) for biotin activity determination in the raw materials for fermentation with active microorganism *Saccharomyces cerevisiae* and method for the microbiological analysis of selected nutrients given in AOAC International (1996) with active microorganism *Lactobacillus plantarum.* Microbiological method by Danielsen and Eriksen (1968) with active microorganism *Saccharomyces cerevisiae* for biotin determination in sugar beet molasses came out accurate, precise, with the detection limit of 0.0100 μg/cm^3 or 10 ppm, because it is the lowest concentacion of biotin which still shows linear response and fits into linear dependence of biotin concentrations - growth area of the yeast. For these reasons this method was accepted as the standard method for determination of biotin in sugar beet molasses by the Institute for Standardization of Serbia.
Key words: *biotin determination, sugar beet molasses, high pressure liquid chromatography, thin layer chromatography, Lactobacillus plantarum, Saccharomyces cerevisiae.*

INTRODUCTION

There are a number of analytical methods for biotin determination which were developed because of importance of biotin and its presence in various mediums (biological fluids, food, pharmaceuticals containing biotin, raw materials). Contemporary analytical methods provide fast biotin determination with low detection limits (Livaniou et al. 2000).
Already published analytical methods for biotin determination can be classified as: microbiological methods, biological methods (bioanalysis), physico-chemical methods and binding assays.
Microbiological methods are the oldest methods for biotin determination, its homologues and derivatives. For microbiological analysis of biotin a number of microorganisms are used: *Lactobacillus plantarum, Lactobacillus casei, Saccharomyces cerevisiae, Ochromonas dani, Micrococcus sodonen, Neurospora crasa, Rizobium trifolii, Escherichia coli 162 (*Livaniou et al., 2000). From the tested microorganisms the most widely used are *Lactobacillus plantarum* and *Saccharomyces cerevisiae*.
Method with active microorganism *Lactobacillus plantarum* even today is widely used for biotin determination in baby food (Watanabe i Fukui, 1996), as well as in molasses (Szopa, 1982, Lončar i sar., 2005, Došenović, 2004).
The yeasts are broadly used in microbiological methods for quantitative determination of "total biotin": biotin, biotin-d-sulphoxide, biocytin, dethiobiotin and pelargonic acid. In molasses it was determined by the yeast *Saccharomyces cerevisiae* (Szopa, 1982; Meledina et al., 1987; Danielsen and Eriksen, 1968; Došenović, 1993; Došenović et al., 1995 and 1995a; Pejin et al., 1996 and 1996a).
Microbiological determinations are easy, although requiring time, and are suitable for routine analysis. They belong to the group of the most sensitive methods for biotin because of their detection limit of 0.1 ng.

Biological methods (bioanalysis) are mostly used for determination of bio-available biotin present in food samples (Bonjour and Machlin, 1984). They are simple but less sensitive than microbiological methods (0.5 ng).
Physico-chemical methods for determination of biotin were developed in the mid- sixties of the 20th century. Application of these methods is limited by their sensitivity. They are mostly used for measuring vitamins in samples with high concentration of biotin, like pharmaceutical multivitamin preparations.
These methods include: spectrophotometry, colorimetry, polarography, thin layer chromatography, gas chromatography, liquid chromatography, high pressure liquid chromatography and capillary electrophoresis.
High pressure liquid chromatography (HPLC) is very important technique for biotin analyses in natural and pharmaceutical preparations. Several methods of HPLC for these determinations are described in the literature (Frappier and Gandry, 1985, Livaniou et al., 2000, Ekpe and Hazen, 1998).
Method RP-HPLC with post-column derivatization is used for biotin determination in food (beef liver, formulations for children, cereals, fruit juices, tomato, multivitamin tablets). The detection limit is about 0.004 mg of biotin without difficulties and with satisfactory reproducibility (Lahely et al., 1999).
Binding assays – the first developed binding assays for biotin determination were radioactive. Radioactive isotopes were used as markers. These methods were used for biotin determination in various samples, like nutrients, extracts of tissues and fluids, particularly human (Kneale and Hood, 1992).
Non-radioactive binding assays for biotin determiation were developed later and they represent progress, because these methods use non-radioactive markers like enzymes, chemiluminescents, bioluminescents, fluorescent substances, latex agglutinative particles, while maintaining high sensitivity (Livanion et al., 2000).
One of the binding assays, very popular and much used nowadays, is ELISA method (Enzyme-Linked Immunosorbent Assay) (Kynng et al., 1998).

Biotin determination in molasses

Sugar beet molasses, which is used in Serbia in the production of baker's yeast, is mainly deficient in biotin content, and that is the reason why in factories a routine and inexpensive method for determination of biotin in molasses is required. Knowing the content of biotin in molasses, the minimum amount of D-biotin or DL-destiobiotin which should be added to molasses to get the maximum yield of yeast and yeast optimum quality can be calculated.
Danielsen i Eriksen (1968) described a simple method for determination of biotin activity in molasses using their own productive yeast as a test organism. Solid substrate without biotin was inoculated with yeast and in the apertures, impressed in the substrate, a series of standard solutions of D-biotin and a series of diluted solutions of molasses were added. After incubation, the zones of growth of yeast were measured and from their diameters biotin content was calculated with a standard error of determining of 0.01µg/g of molasses.

MATERIAL AND METHODS

Biotin was determined in decadal samples of sugar beet molasses from four local sugar factories by method of high pressure liquid chromatography (HPLC), thin layer chromatography (TLC), microbiological method by Danielsen and Eriksen (1968) with active microorganism *Saccharomyces cerevisiae* (commercial baker’s yeast from two domestic producers) and microbiological method given in AOAC International (1996) with active microorganism *Lactobacillus plantarum* (ATCC 8014 from collection E. Merck, Darmstadt, Germany). Applied methods are described in detail in Došenović’s PhD thesis (2004).

RESULTS AND DISCUSSION

Determination of biotin by HPLC method

Strong base ion exchanger LEWATIT MP 500A is very efficient in purification of "raw biotin". Eluates are light yellow and it is confirmed by RP-HPLC analysis of standard solution of biotin (B_k) that there is no biotin retention. Nevertheless, the retention periods of biotin transmitted only trough column (B_k) and of biotin treated with various types of active coal (B_A, B_{A1}, B_{A2}) are to some extent shorter from the retention period of standard of biotin (B) – solution of biotin which is directly injected in column of chromatograph (Table 1). Probably some changes with biotin are made during transmission trough ion exchanger. Although the peaks of the different biotin samples have similar intensity on wavelength of 230 nm and 220 nm (Figure 1), there are some differences in their retention periods (Table 1)

Table 1. Retention periods of various samples of biotin on different wavelenght

Biotin sample*	Retention period T (sec)	
	λ=220nm	λ=230nm
B	313	308
B_k	251	248
B_A	306	248
B_{A1}	306	306
B_{A2}	306	306

**B-basic solution of biotin; B_K-biotin transmited trough column; B_A, B_{A1}, B_{A2}-sequentially biotin treated with powder, spiked and granulated active coal*

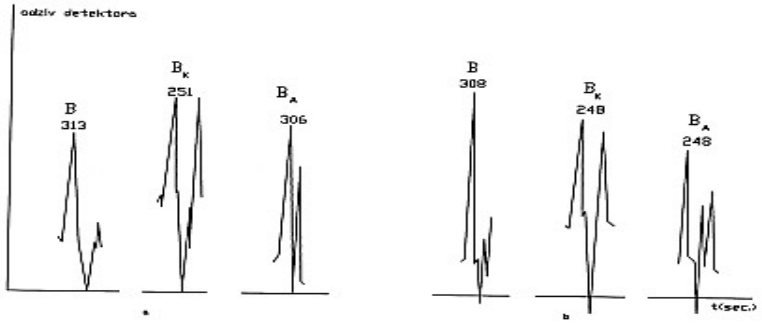

Figure 1. HPLC chromatogtam of various samples of biotin with UV detection on 220nm (a) and 230nm (b); Marks are like in Table 1.

From eluates of molasses, only the sample M_4 was analysed with RP-HPLC method, and it was the brightest. Chromatogram has the shape of stretched peak with retention period of about 15 minutes. That shows that the eluate of molasses, even prepared by complex process and visually acceptable, is not sufficiently pure for analysis with the applied method.

Determination of biotin by TLC method

Since the HPLC method was not able to identify biotin in molasses, further examinations were continued with the thin layer chromatography (TLC) with silica gel layer and water as mobile phase. The retention values of different samples of biotin and biotin from molasses (M_4) are shown in Table 2.

Biotin from molasses can be extracted on granulated active coal with granule size of 2.5 mm and be identified on thin layer of silica gel. Extraction and determination of biotin from molasses by the applied procedure is very complicated. Adsorption and desorption of biotin, evaporating the solution after desorption and after passing through a column last very long. Neither is adsorption selective enough, nor is the extraction of "raw biotin" on strong base ion exchanger efficient enough for the quantitative analysis of biotin. The final solution is not sufficiently clear, which disables the determination of biotin in molasses by liquid

chromatographic methods. In addition, chlorine-tolidine reagent, although with a detection limit of 0.2µg of biotin (Frappier and Gaudry, 1985) it is not specific only for biotin.

Table 2. R_f values of different samples of biotin and biotin from molasses

Sample	R_f x 100
B	65
B_k	83
B_{A2}	82
M_4	81

M_4-sample of molasses 1 from granulated active coal and electric vibrator; other marks are like in Table 1.

Determination of biotin by microbiological method – with active microorganism *Saccharomyces cerevisiae*

The precision and accuracy of method are examined and deviation results, expressed as % of utilization or recovery in relation to additional real value of biotin, are given in Table 3.

Table 3. Results of deviation from the true value of biotin content

Biotin added µg/cm^3	Biotin detected µg/cm^3	Utilization %
0.0400	0.0385	96.25
0.0800	0.0795	99.38
0.1200	0.1190	99.17

As seen from Table 3 the efficiencies are above 95% biotin indicating that the applied microbiological method for determination of biotin in molasses commercial yeast *Saccharomyces cerevisiae* is a very accurate analytical method.

Figure 3 shows linear dependence of diameter of growth zone of yeast on biotin concentration in standard solutions added into substrate.

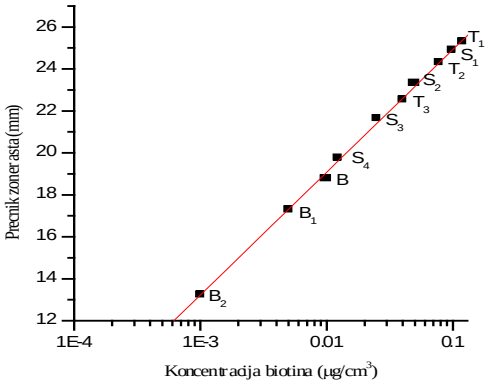

Figure 3. Dependence of diameter of growth zone of yeast on the concentration of biotin in the substrate (marks are like in Table 1 and 3)

From figure 3 it is obvious that the linearity is excellent in the concentration range of biotin 0.001-0.1200 µg/cm^3. The correlation coefficient of linear dependence is 0.99943. This range is sufficient for our analyses since the biotin content in the tested molasses is from 0.02951µg/g to 0.08465 µg/g of molasses.

For determination of the detection limit the following solutions of D (+)-biotin are prepared: B-10ng/cm^3. B_1-5 ng/cm^3. B_2-1 ng/cm^3. B_3-0.5 ng/cm^3. S_3-25 ng/cm^3. S_4-12.5 ng/cm^3.

The results of the entire experiment are shown in Table 4.

Table 4. Zone diameters of growth of yeast depending on the concentration of biotin in biotin standard solutions

Biotin solutions ng/cm^3	S_3 25	S_4 12.5	B 10	B_1 5	B_1 5	B_2 1	B_2 1	B_3 0.5
I Petri dish								
R. mm	21.50	20.00	19.20	16.50	16.50	13.50	13.10	+
	21.00	20.50	19.10	17.00	16.50	13.50	13.10	+
	21.50	19.50	19.50	17.50	17.00	13.00	13.50	+
	21.50	20.10	19.50	16.50	17.10	13.10	13.00	+
II Petri dish								
R. mm	21.50	19.20	18.20	17.50	18.00	13.00	12.50	+
	21.50	19.30	18.30	17.50	17.50	12.50	13.00	+
	21.40	19.30	18.20	17.50	17.10	12.50	13.00	+
	21.50	19.20	18.50	18.00	17.50	12.50	13.50	+
III Petri dish								
R. mm	22.20	20.30	18.50	17.10	18.10	14.00	14.00	+
	21.50	20.50	19.10	17.10	17.50	13.50	14.00	+
	21.50	21.00	19.20	17.50	17.50	13.50	13.20	+
	21.50	20.60	18.50	17.50	18.10	13.60	13.50	+
mean R. mm	21.51	19.91	18.81	17.27	17.37	13.18	13.28	

R - zone diametar of growth of yeast

The presented results indicate that the detection limit of biotin is 0.5 ng/cm^3. At that concentration of biotin, observed with the naked eye, a very weak whitish irregular zone of yeast growth was hardly noticed and the diameter was not possible to measure. In Table 4 the presence of yeast growth at this concentration of biotin is marked with plus (+).
According to the results the concentration of biotin of 0.0100µg/cm^3 or 10 ppm could be used for the limit of determination because this is the lowest concentration of biotin which still shows linear response and fits into linear dependence of biotin concentrations - growth area of the yeast.

Determination of biotin by microbiological method – with active microorganism *Lactobacillus plantarum*

In comparison with biotin content in molasses 2 and 3 determined by the yeast *Saccharomyces cerevisiae* (Table 5 and 6) significantly higher content of biotin is obtained with *Lactobacillus plantarum* (Table 7). This is not expected since the growth of *Lactobacillus plantarum* is stimulated only by the D (+)-biotin and the growth of *Saccharomyces cerevisiae* besides biotin also by some of its analogues. The colour of solution certainly had influence on the results as at nephelometric measurement (intensity of scattered radiation (IRZ) and transparency (%T)). Furthermore, no research on the presence of other stimulating substances for growth of *Lactobacillus plantarum,* such as niacin and tryptophan, was made (AOAC. 1996).

Table 5. Biotin contetnt in molasses determined by commercial yeast. A" and calculated with graphic method and with Mathcad package (mcd)

Molasses	No. of mesurement	$\overline{x}$.µg/g	s	s^2	CV. %	F-criteria
2	8	0.04027	0.004132	0.00001707	10.26	2.14
2(mcd)	8	0.03838	0.006046	0.00003655	15.75	
3	8	0.03390	0.004256	0.00001812	12.55	8.04
3(mcd)	8	0.03462	0.012070	0.00014570	34.86	

$\overline{x}$ *-average biotin content. s-standard deviation.* s^2*-variance.CV- coefficient of variation*

Table 6. Biotin content in molasses determined by commercial yeast „B" and calculated with graphic method and with Mathcad package (mcd)

Molasses	No. of mesurement	$\overline{x}$.µg/g	s	s^2	CV. %	F-criteria
2	4	0.05583	0.004039	0.00001632	7.23	16.32
2(mcd)	4	0.05750	0.001000	0.00000100	1.74	
3	4	0.07365	0.004585	0.00002102	6.22	2.05
3(mcd)	4	0.07950	0.006557	0.00004300	8.25	

Table 7. Biotin content in samples of molasses 2 and 3 determined by measurement of transparency using different mediums and biotin standards

Biotin (µg/g of molasses)/solution volume (cm^3)								
	standard 0.14ng/cm^3				standard 0.18ng/cm^3			
Samples of molasses	medium LP2							
	1	2	3	4	1	2	3	4
2	0.160	0.111	0.092	0.088	0.120	0.088	0.074	0.070
3	0.230	0.160	0.130	0.120	0.170	0.120	0.100	0.090
Samples of molasses	medium "Vitamin-Biotin Testbouillon							
	1	2	3	4	1	2	3	4
2	0.190	0.105	0.083	0.071	0.125	0.083	0.066	0.062
3	0.220	0.145	0.122	0.104	0.175	0.142	0.103	0.092

CONCLUSIONS

In this paper the advantages and disadvantages of applied analitical methods for determination of biotin in sugar beet molasses are shown. in order to point the importance of use of microbiological method with active microorganism *Saccharomyces cerevisiae*.
The applied method for determination of biotin with *Saccharomyces cerevisiae* is accurate. precise. with the limit of quantitative detection of 0.0100 µg/cm^3 or 10ppm.
Therefore it can be recommended for operational control of the quality of molasses used in the production of baker's yeast or for the determination of biotin - a growth factor for this yeast - in molasses.
This method was accepted by the Institute for Standardization of Serbia as Serbian standard SRPS E. L8. 021: 2006. under the name Molasses – Determination of Biotine content.

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EXPERIMENTAL MODELS TO STUDY THE IMPACT OF FOOD-RELATED CONDITIONS ON VIRULENCE PROPERTIES OF POULTRY MEAT ISOLATE *Campylobacter jejuni*

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ABSTRACT: *Campylobacter jejuni* has become the leading cause of human inflammatory enteritis and of the most important zoonosis worldwide. Despite of its high prevalence, extraintestinal survival mechanisms and virulence properties are still poorly understood, also due to the lack of useful models for such studies. We confirmed previously bacterial environmental stresses to be implicated in the adhesion, invasion and intraepithelial survival of *C. jejuni* in Caco-2 cells, J774 murine macrophages and PSI cell lines in *in vitro* cell models. In this work BALB/c mice were experimentally infected with stressed *C. jejuni* cells to mimic the infection *in vivo*. After bacterial spreading and tissue invasion we followed the survival of stressed campylobacters (exposed to starvation or short-term oxidative stress) at 1, 3 and 8 days post infection in the organs (liver, spleen) of infected animals. Colonization of untreated as well as stressed campylobacters can occur in the livers and spleens of BALB/c mice. Starvation impaired the ability of *Campylobacter* to persist in organs, however, short term exposure to oxygen enhanced its survival and duration of infection. Thus we confirmed in *in vitro* and *in vivo* model that adaptive stress response is crucial for *Campylobacter* survival during host-pathogen interaction and its pathogenicity.
Key words: *Campylobacter jejuni, environmental stress, bacterial virulence, cell monolayers.*

INTRODUCTION

Campylobacter jejuni is the most common bacterial cause of food-borne disease and the leading cause of diarrheal disease in humans (Allos, 2001). This microaerophilic, highly motile, gram-negative rod-like bacterium with an optimal growth temperature at 42 °C is part of the normal intestinal flora of many domestic and wild animals, with poultry as a major source of human infection. It is highly infectious and infective doses as low as 500 to 800 CFU have been reported. The clinical symptoms vary significantly from mild watery diarrhea to severe enteritis with bloody diarrhea, abdominal cramps, fever, and nausea (Janssen et al., 2008). In majority the disease is mild and self-limiting, but extra-intestinal post-infection complications, e.g. Guillain-Barre´ syndrome (GBS) and reactive arthritis can occur (Zilbauer et al., 2008).
Although *C. jejuni* is very susceptible to environmental stress conditions due to the lack of stress-adaptive response factors such as RpoS, CspA, OxyR, and SoxRS it has developed survival mechanisms, both inside and outside the host. Exposure to unfavorable environmental conditions may trigger conversion of *C. jejuni* into a viable but nonculturable (VBNC) state (Klančnik et al., 2009). Despite the significant health burden caused by the organism, the mechanisms of pathogenesis and host immunity response during the course of disease are not yet well understood. Following the passage through the acidic environment of the stomach, surviving bacteria adhere to intestinal epithelial cells or to the mucus overlying these cells and replicate in both small and large intestine. Motility and adhesion of

C. jejuni are essential steps for human disease and colonization in animals. Disease development involves a multifactorial process requiring bacterial adherence to host cells, epithelial cell invasion and replication, secretion of virulence proteins, and bacterial translocation across the epithelial cell barrier of the intestine (Konkel et al., 2001; Park, 2002). Once, reaching the underlying tissues *C. jejuni* is no longer exposed to peristaltics, interacts with professional phagocytic cells and can cause systemic infection, which is reflected in occasional recovery from spleen and liver of infected host. Colonic biopsies from infected patients show an acute inflammatory response with infiltration of the epithelium and lamina propria with neutrophils and mononuclear cells, crypt abscesses, focal ulcerations, and plasma cell proliferation (Parathasarathy and Mansfield, 2009, Berreswill et al, 2011). Furthermore, the ability of bacteria to survive within phagocytic cells represents a strategy to evade the host defences and migrate to other areas of the body. Previously, we have demonstrated that despite being efficiently internalized, *Campylobacter* was eliminated from murine macrophages within 24h to 30h post infection (Šikić Pogačar et al, 2009). This can explain why bacteremia occurs very rarely (Allos, 2001).

Various models to study the pathogenesis of *Campylobacter* were established. Polarized or nonpolarized epithelial cell culture as well as animal models can be used to study mechanisms of *C. jejuni* infection. Cell culture models have shown the ability to adhere, invade and translocate across intestinal epithelial cells. Large animal models and ferrets enabled early progress in studies of pathogenesis, transmission, and vaccines. Chickens, ferrets, dogs, primates, rabbits, mice, and pigs have been inoculated experimentally by various routes to mimic the course of infection in humans (Mansfield et al., 2008). Even, larvae of Galleria mellonella (Greater Wax Moth) have been recently shown to be susceptible to *C.jejuni* infection and were used as infection model (Senior et al., 2011). Monkeys are the best model, but expensive and not available to most institutions. An optimal animal model should assure: symptoms similar to those observed in human cases following oral administration of bacteria, availability, ease of manipulation, repeatability, inexpensive, and also known immune status of animal (Mansfield et al., 2008). However, animals are generally silent carriers and thus symptoms do not always appear. In addition, animal should have a sufficient life span to complete the entire study (Haddad et al., 2010). ‘‘Complete’’ models, which reproduce intestinal disease induced by *Campylobacter* (particularly diarrhea), must be distinguished from ‘‘partial’’ models, in which only one or a few aspects of virulence (e.g., colonization, adhesion, internalization, or toxigenesis) are reproduced (Haddad et al., 2010).

In humans, disease symptoms and course of infection can vary dramatically and the outcome depends on many factors, such as the dose and virulence of *C. jejuni* strain, the immune status of host, the composition of patient’s entric microflora etc. Despite the difficulties, pathophysiological findings from *in vivo* studies using animal models complement *in vitro* studies and provide a deeper insight in the course of human infection. For this reason the present article focuses on confirmation of the results from *in vitro* cell culture models with *in vivo* BALB/c mouse model study used to investigate colonization and survival of bacterial cells during infection.

MATERIAL AND METHODS

Bacterial strain, culture media, growth and stress conditions

A poultry meat isolate *C. jejuni* K49/4 was previously identified, stored at -80 °C and subcultured prior to subjecting to test conditions as described previously (Klančnik et al., 2009). For starvation, the cells were harvested by centrifugation (12000xg, 5 min, 4 °C), washed, resuspended in Ringer solution (KH2PO4, 5 mM, Kemika), and incubated 24 h microaerobically at 42 °C. For oxidative stress, the cells were exposed to oxygen at atmospheric concentration for 5 hours. Untreated *C. jejuni*, taken from culture at the same time as the stressed organisms, were used as controls.

In vitro cell lines and growth conditions

Caco-2 cells and J774 macrophages were grown in Dulbecco's minimum essential medium (DMEM) and RPMI 1640 medium as described previously (Šikić Pogačar et al., 2009, Rubeša Mihaljević et al., 2007)

Adhesion, invasion and intracellular survival

For all assays, 24-well tissue culture trays were seeded with approximately 5.0 $x10^5$ cells per mL and incubated for 24 h. The bacterial number was determined spectrophotometrically (absorbance mode at a wavelength of 600 nm). Infection was carried out by inoculating *C. jejuni* at an approximate MOI of 100. Infected monolayers were incubated for 2 h to allow adhesion and invasion as described previously (Šikić Pogačar et al, 2009). The monolayers were washed with medium without antibiotic to remove unbound bacteria and medium containing 100 mg per mL of gentamicin for enumeration of intracellular bacteria. The monolayers were lysed with cold distilled water and released intracellular bacteria were enumerated by the culturability assay 24, 72 and 96 h post infection. The total number of adherent and internalized bacteria in macrophages was determined simultaneously by performing an invasion assay without gentamicin treatment. The difference between the total and intracellular bacteria was calculated to obtain adherent *C. jejuni* cells. Results are expressed as the mean of the log10 CFU/mL of adherent or internalized bacteria for three independent measurements.

Polarized epithelial cell model of PSI cl.1 cells

To obtain polarized epithelial cell monolayers, we seeded PSI cl 1 cells onto Transwell® filter inserts and infected them with *C. jejuni* to allow adhesion and invasion as described previously (Šikić Pogačar et al, 2010).

In vivo experiments

Male BALB/c (H-2d) mice 8- to 12-weeks-old were used in all experiments and infected intravenously with a single dose of 0.5-1$x10^9$ CFU/mL starved or untreated *C. jejuni* cells. After 24, 72 and 96 h post infection the number of *C. jejuni* CFU in livers and spleens was determined as previously described (Vučković et al., 1998). The experiments were repeated 3-5 times. Data are presented as the $\log_{10}$ of the mean ± SD bacterial CFU per organ.

Statistical analysis

Data on bacterial counts from different experimental groups were compared using the One-way ANOVA and Tukey-b test for post hoc analysis. All statistical values were considered significant at the P level of 0.05. Statistical analysis was performed using Statistica for Windows version 6.1 (Statsoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Influence of enviromental stresses on virulence properties of *Campylobacter jejuni* in *in vitro* cell culture model

The virulence of the *C. jejuni* K 49/4 as indicated by the ability of bacteria to adhere to, invade, and replicate within different cell lines was investigated. Intestinal epithelial cells: colon adenocarcinoma human cells Caco-2, mature small intestinal pig cells PSI and murine macrophages J774 were used in the study as *in vitro* models of infection. We examined the influence of 5 h starvation and 5 h oxygen exposure on the ability of *C. jejuni* to adhere, invade, and replicate (survive) within cell cultures measured by plate-count assay and compared to a non-stressed control. Our results showed that starvation reduced *C. jejuni* ability to adhere to the surface of eucaryotic cells and invade these cells (Figure 1). On the other hand, short oxidative stress lasting for 5 h improved *C. jejuni* virulence properties, improved bacterial ability to adhere and invade cell cultures (Figure 1).

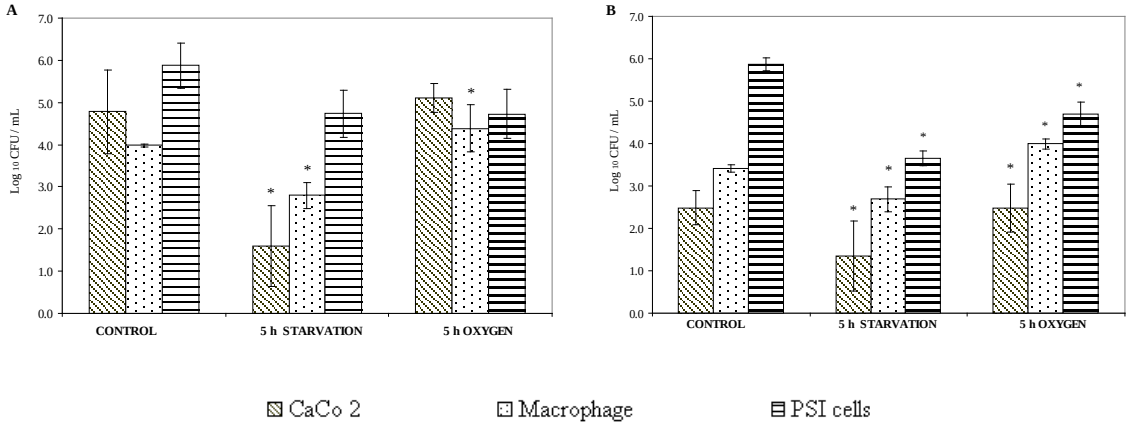

Figure 1: Adhesion (A) and invasion (B) ability of control, 5-h-starved and 5-h-oxygen exposed *C. jejuni* in Caco-2, macrophage and PSI cell lines.

Starvation also reduced *C. jejuni* ability to survive within cell cultures. Even though, both, control and starved *C. jejuni* survived intracellularly during the entire observation period in Caco-2 cells and PSI cl.1 cells. The number of recovered starved cells remained high throughout the entire experimental period, but did not reach the control level. A significant proportion of starved bacterial cells persisted within Caco-2 cells even after 5 days. In addition, the number of non-stressed (control) bacteria within Caco-2 cells started to decrease 72 h post infection, 48 h post infection in case of 5 h starvation, while on the other hand, the number of bacteria exposed to atmospheric oxygen prior to infection increased until 72 h post infection, when it slowly started to decline (Figure 2A).

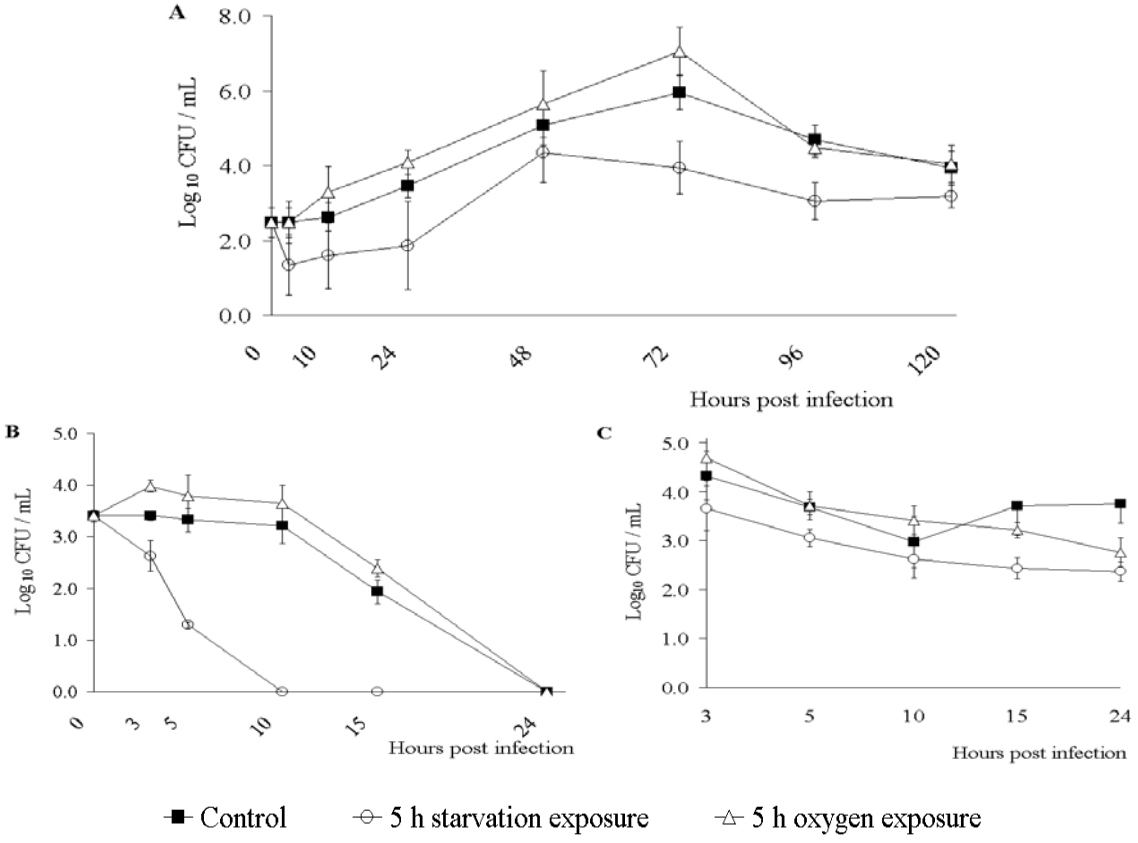

Figure 2: Intracelullar survival of control, 5-h-starved and 5-h-oxygen exposed *C. jejuni* in Caco-2 (A), macrophage (B) and PSI (C) cell lines.

Survival pattern of *C. jejuni* within murine phagocytes J774 exhibited similar survival characteristics, and already 5 h post-infection showed a progressive decrease in the number of intracellular bacteria. No intracellular bacteria were recovered 29 h post infection, indicating extensive killing by the macrophages. However, considerable differences in the number of intracellular bacteria were observed. Bacterial starvation once again significantly decreased the survival period of bacteria within J774 macrophages while 5 h pre-exposure to atmospheric oxygen enhanced its intracellular survival (Figure 2B).
The decrease in the number of survived bacteria within polarized PSI cl. 1 cells was observed 5 h post- infection in case of 5 h starvation. On the other hand, bacteria exposed to 5 h oxygen stress were recovered in the same number as control 5 h-post infection. Furthermore, 10 h post-infection bacterial count exceeded the number of control, thereafter the number of bacteria exposed to oxygen prior to infection began to decrease gradually and remained below control level (Figure 2C).
Our results are consistent with the study by Day et al. (2000), confirming the ability of *C. jejuni* to survive intracellularly for relatively long periods both in phagocytes (72 h) and in intestinal epithelial cells (96 h), persuading some investigators to classify this organism as a facultative intracellular pathogen. The role of macrophages in development *C. jejuni* enteritis is still unclear. There exist contradictory reports regarding the ability of *C. jejuni* to survive within macrophages, which may be the consequence of the use of phagocytic cells of different origin and various bacterial isolates showing different virulence potential. However, any prolongation of survival in macrophages may increase the probability of transmission of bacteria in the host organism and have further implications in the pathogenesis of campylobacteriosis.
Although the results obtained from cell cultures provide information on survival of *C. jejuni* under stress conditions and influence of stress on its virulence properties, they might not truly reflect the situation *in vivo*. For that reason, we used animal model of BALB/c mice to validate *in vitro* cell model of testing virulence properties of *C. jejuni.*

Influence of enviromental stresses on virulence properties of *Campylobacter jejuni* in *in vivo* BALB/c mice model

Different patterns of bacterial growth were observed in spleens and liver of BALB/c mice. The results of *in vivo* experiments in mice confirmed impairment of *C. jejuni* virulence properties upon starvation (Fig 3A and B).

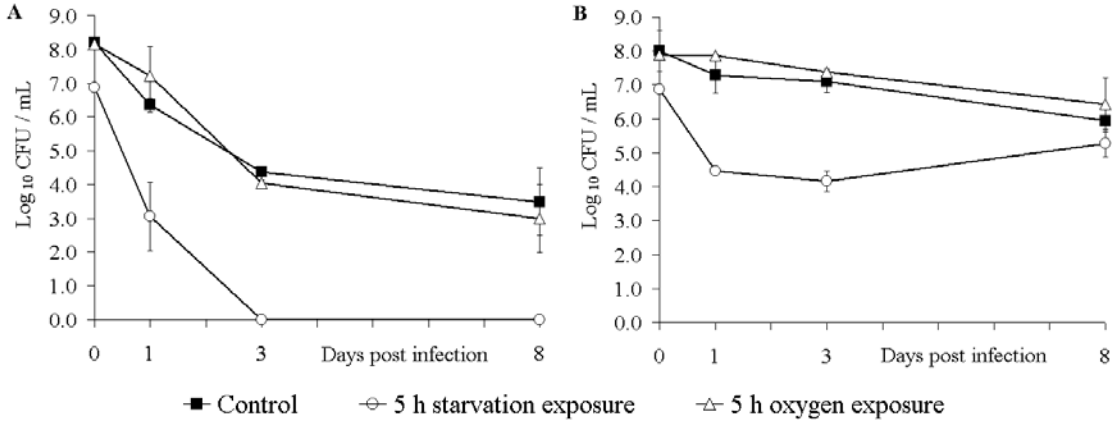

Figure 3: Intracelullar survival of control, 5-h-starved and 5-h-oxygen exposed *C. jejuni* in spleen (A) and liver (B) of mice.

The number of isolated bacteria from spleens was significantly lower and the infection was resolved as early as three days post infection (Figure 3A). In the livers, a marked difference in the number of isolated bacteria was seen, but the duration of infection was similar regardless of starvation (Figure 3B). 5h-atmospheric oxygen exposure improved the ability of *C. jejuni* to persist in liver and showed better survival pattern than non-stressed *C. jejuni.*

CONCLUSIONS

Since *C. jejuni* is mainly a food-borne pathogen, stress factors in the food chain may have an impact on its viability in the product and are important risk for human health. This study demonstrated that cell culture-based virulence of *C. jejuni* was generally equivalent to the infectivity in a BALB/c mouse model. The results showed that starvation reduces the ability of *C. jej*uni to survive within polarized and non-polarized cell cultures lines as well as in mouse model of infection. Like in cell culture based assays, short exposure of *C. jejuni* to atmospheric oxygen concentration enhanced its ability to survive and persist within animal host.

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QUALITY OF ALTERNATIVE OIL AND PROTEIN CROPS

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ABSTRACT: The most important oil and protein plant crops belong to different families. Constant increase in demand for plant oils used as food or for processing industry inspired evaluation of other, insufficiently used plant species for breeding and commercial production. Fifteen plant species were grown during 2010 on a quarantine plot of the Institute of Field and Vegetable Crops in Novi Sad. Oil and protein content was determined in the seeds accordingly. The highest oil content was found in castor oil plant (52.28%), and the lowest in chard (4.58%). Protein content was highest in white mustard (39.54%), and lowest in castor (13.12%), while the largest total content of oil and protein was found in oil gourd (82.62%), and the lowest in chard (21.94%). Selected species will be included in further research as a source of oil and protein for various purposes.
Key words: *protein content, oil content, alternative crops*

INTRODUCTION

The demand for plant oils used for food and feed or for processing industry is in constant increase (Popović et al., 2010). In order to make plant oil production profitable, cultivated plants should have high oil content in the plant parts used for its extraction. The residues remaining after pressing are commonly used as a source of plant protein used in animal nutrition, but also in the food industry. The high protein content and their quality ensure nutritional value, and thus the market value of these nutrients.
Changes in the global oil market, demand for oil of specific quality, growing requirements of cultivated plants and their exposure to different biotic and abiotic stresses, require more research on under-used cultivated plant species.
Unlike the cultivated cereals, oilseed plant species belong to different and often phylogenetically very distant families and genera. This origin also requires diversity in the study methods of qualitative and quantitative characteristics and application of plant breeding methods.
In order to investigate quality of under-grown oil-protein plant species from the collection of the Institute of Field and Vegetable Crops in Novi Sad, oil and protein content in 15 plant species was analysed.

MATERIAL AND METHODS

The Department of Oil Crops of the Institute of Field and Vegetable Crops in Novi Sad holds a collection of 15 oil plant species represented with different number of accessions. The collection includes: flax (*Linum usitatissimum* L.), false flax (*Camelina sativa* Crtz.), castor oil plant (*Ricinus communis*), safflower (*Carthamus tinctorius* L.), sesame (*Sesamum indicum* L.), mary thistle (*Silybum marianum* syn. Carduus Marianus), caper spurge (*Euphorbia lathyris* L.), coriander (*Coriandrum sativum* L.), dill (*Anethum graveolens* L.) and okra (*Hibiscus esculentus*).
The collected species were of different geographical origin and the collection was formed through a long period by exchange with other scientific research organizations.

Optimal agro-technical practices were applied for the cultivation of each plant species (Todorovic et al., 2003). Harvesting was carried out when the seed was at the technological maturity. Seeds were used for the analysis of oil content in all tested species, except for the caper spurge where tubers were analyzed.
Oil content was analyzed by classical Soxhlet method according to the ISO standard 659:1994, involving gravimetric determination of oil using light petroleum extract from oilseeds. The petroleum extract is called “oil content”. Protein quantity was determined using classical micro-Kjeldahl method, on VAP-50, Gerhardt apparatus, in the Chemical Laboratory of the Department of Oil Crops of the Institute of Field and Vegetable Crops in Novi Sad.

RESULTS AND DISCUSSION

The tested plant species differed widely in oil content. Species with high contents were allocated to one group: castor oil plant (52.28%), caper spurge (50.08%), oil gourd (47.87%), flax (39.95%) and false flax (35.69%) (Figure 1).

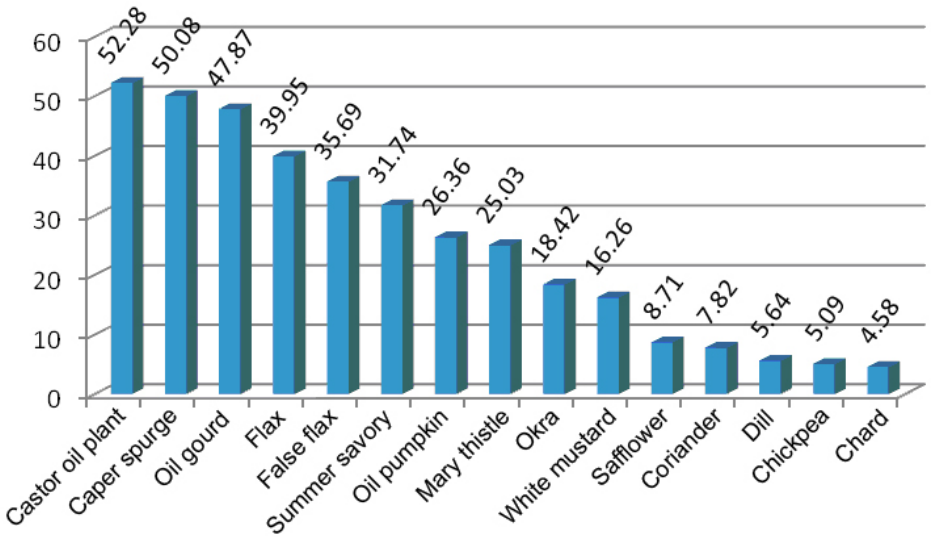

Figure 1. Oil content of alternative oil plant species (%)

Toxic compounds in the oil of castor oil plant and caper spurge present a problem for its use (Knights et al., 2001), therefore the oils obtained from these species can only be used as raw materials for industrial processing.
The group with medium oil content consisted of savory (31.74%), oil pumpkin (26.36%), mary thistle (25.03%), okra (18.42%) and white mustard (16.26%). Although these species hadslightly lower oil content, they hadgood prospects for the production of high quality oil (Knights et al. 2001). It can be used for human consumption, mostly as cold pressed oil, or in the food and pharmaceutical industries.
Due to its low boiling point, pumpkin oil is not suitable for frying, but is used as salad oil, and because of healing powers it is applied in pharmaceutical industry (Berenji, 2011).
Mary thistle oil is extremely rich in tocopherols, which is why it is recommended for human consumption (El-Mallah et al., 2003). White mustard contains oil high in erucic acid and as such is used for industrial purposes (Zheljazkov et al., 2012).
Low oil content group is formed of safflower (8.71%), coriander (7.82%), dill (5.64%), chickpea (5.09%) and chard (4.58%). The low oil content found in safflower is different from the values given by other authors (Beaulieu et al., 2009), which can be explained as a result of the used testing method. In our research we analyzed the entire fruit-seed, not just the core. The high content of shell significantly reduced the value of the resulting oil content. In addition, safflower is a plant species of arid climate and of soil below the natural fertility, while conditions in our experiment were not fully optimal for this plant species.

Based on the protein content, plants species were also divided into three groups. The highest protein content was found in white mustard (39.54%), oil gourd (34.75%) and false flax (28.78%). The mean content had flax (24.17%), savory (23.59%), chickpea (23.19), oil pumpkin (22.98%) and okra (22.73%) (Figure 2).

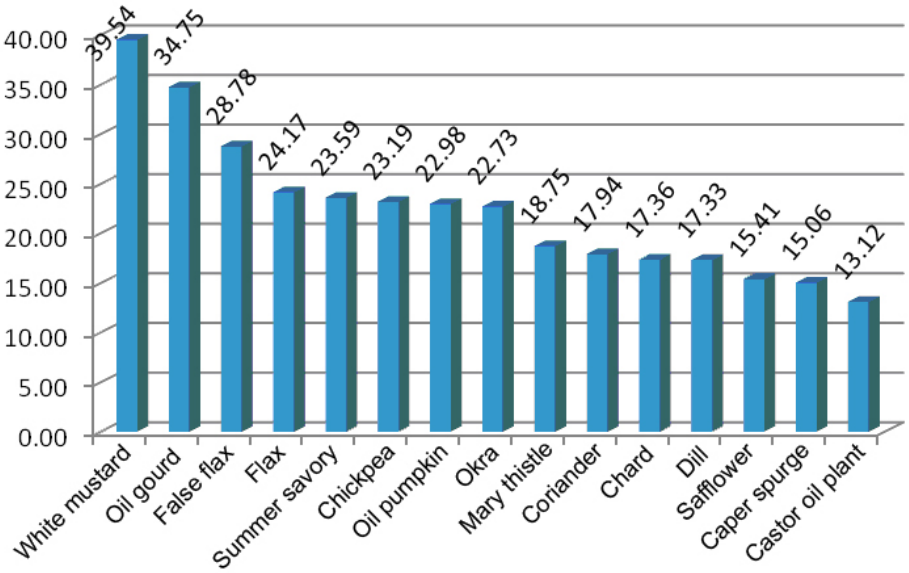

Figure 2. Protein content of alternative oil plant species (%)

The lowest contents were found in mary thistle (18.75%), coriander (17.94%), chard (17.36%), dill (17.33%), safflower (15.41%), caper spurge (15.06%) and castor oil (13.12%).
The low protein content of castor oil plant and caper spurge is expected due to the high oil content. A large number of authors found a negative correlation between these two properties with other oil crops (Chung et al., 2003; Zhao et al., 2006).
Oil gourd (82.62%), false flax (64.47%) and flax (64.12%) have a high content of both components in the seeds (Figure 3).

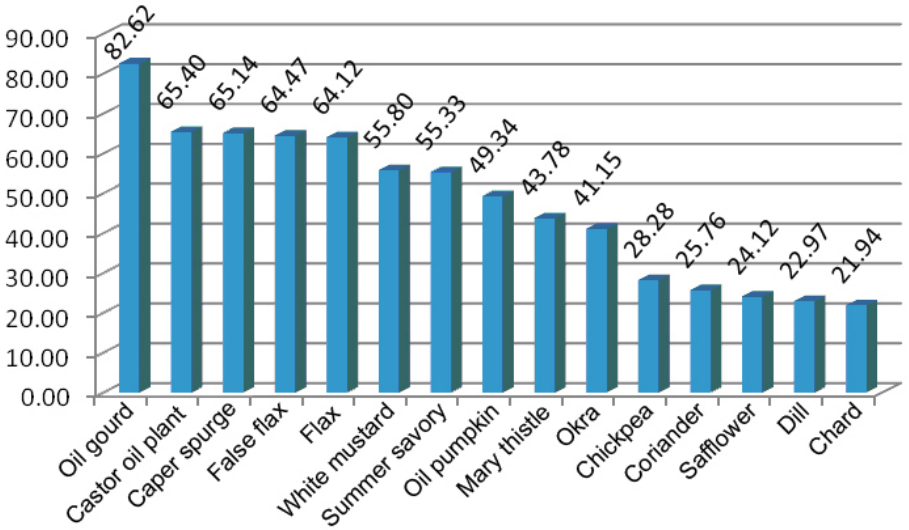

Figure 3. Combined oil and protein content of alternative oil plant species (%)

This allows their use as oil and protein crops. Despite the high total content in castor oil plant (65.40%) and caper spurge (65.14%), using proteins from their seed is not possible because toxic substances are present in large percentage (Griffiths et al., 1987; Fuller and McClintock 1986).

In addition to use as a feedstock for the production of oil or proteins, some species are used in the production of food for domestic animals, especially birds. The total oil and protein content in flax (64.12%), white mustard (55.80%) and safflower (24.12%) are important traits that determine their share in the feeding mixtures as high-energy components.
Commercialization of the studied species requires determination of seed yield, i.e. tubers, to determine the profitability of production.

CONCLUSIONS

Fifteen tested oil-protein species from the collection of the Institute of Field and Vegetable Crops differ in oil and protein content, as well as in total content of both components. Plant species which have great potential to be used as oil plants (castor oil plant, flax, oil pumpkin), protein plants (white mustard, false flax), or protein-oil plants (oil pumpkin, flax, false flax) were selected. Further studies of these species will be focused on requirements of processing industry and the market.

ACKNOWLEDGEMENTS

This work is a result of research under the project TR-31025 funded by the Ministry of Education and Science of the Republic of Serbia.

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SIGNIFICANCE OF INTERNAL AMPLIFICATION CONTROL FOR THE DETECTION OF HUMAN NOROVIRUSES FROM DIFFERENT FOOD SAMPLES BY REAL-TIME RT-PCR

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ABSTRACT: Among human enteric viruses, the noroviruses (NoVs) are of a major epidemiological significance as a common cause of both epidemic and sporadic non-bacterial gastroenteritis in humans. According to the recent CDC data, NoVs caused most illnesses (58%) through consumed contaminated food especially soft fruits, vegetables and RTE foods. The most effective methods for virus detection in food samples are those based on nucleic acid amplification. However, two major obstacles that influence routine virus detection in foods include the low efficiency of concentration and nucleic acid extraction procedures and the presence of inhibitors from food matrix to the molecular reactions. Most false negatives are consequence of inefficient virus and/or nucleic acid extraction and of inhibition of the RT-PCR reaction. To verify whether amplification reactions have functioned correctly, implementation of internal amplification controls (IACs) is essential. For this purpose, previously constructed IACs for application with highly reactive degenerate primers (COG) for the detection of human NoVs genogroups GI and GII by real-time RT-PCR were tested. Twenty-five grams of raspberries, lettuce, cherry tomatoes, green onions and deli meat have been artificially inoculated with norovirus GI and GII positive stool samples. IACs were incorporated in all real-time RT-PCR assays, and simultaneous amplification of both target and RNA IAC was achieved after optimization without decreasing detection levels of virus. In this study it has been shown that IACs amplification was unaffected by the viral RNA extraction procedure from different food items, which could potentially be a source of diverse inhibitors.
Key words: *noroviruses, detection, food, internal amplification control, real-time RT-PCR*

INTRODUCTION

Enteric viral transmission due to the consumption of contaminated foods is clearly recognized as a significant public health concern. Recent estimation revealed that in the US, most foodborne illnesses were caused by noroviruses (NoVs), 58% out of 9.4 million episodes caused by 31 major pathogens (Scallan et al., 2011). In Europe, human NoVs are also the principal cause of foodborne illness (Phillips et al., 2010). Food is a common vehicle for human NoVs transmission due to the contamination with human fecal material, poor personal hygiene of food workers and virus long survival in the environment (Atmar, 2010). Therefore, it is not surprising that the detection of NoVs in food, especially salad vegetables, soft fruits, and ready-to-eat (RTE) has become very important. These food items have been implicated in numerous viral outbreaks (Le Guyader and Atmar, 2008).
Virus detection from food includes diverse challenges, such as typically low viral load, extremely genetic heterogeneity, and presence of food components (e.g., organic and phenolic compounds, glycogen, fats, and Ca^{2+}) that are able to inhibit molecular assays. Although detection of NoV relies on molecular methods, with (real-time) reverse transcription (RT)-PCR considered as the gold standard due to high sensitivity and specificity, it is well known that PCR reactions are susceptible to inhibitors (e.g., humics, complex polysaccharides, microorganisms debris, metal ions, organics, and nucleases) ubiquitous in environmental samples (Julian and Schwab, 2012). Though the identities and biochemical mechanisms of action of many inhibitors remain unclear, bile salts and complex polysaccharides in feces, heme in blood, and urea in urine have all been shown to inhibit

PCR, probably through interference with binding and/or polymerization activity of DNA polymerases (Nolte, 2004). Carryover of reagents used for isolation of nucleic acids from samples can also inhibit amplification reactions. Generally, inhibitory factors that have been identified in PCR reactions included organic and inorganic chemicals, detergents, antibiotics, buffers, enzymes, polysaccharides, fats, and proteins (Wilson, 1997).
The vast number of PCR inhibitors can lead to unacceptable high number of false negatives in real-time PCR detection of NoV contamination in food. Other causes of false-negative results include target nucleic acid degradation, sample processing errors, thermal cycler malfunction, and in reverse transcription-PCR, failure of the reverse transcription step. To eliminate the risk of false negative results it is recommended that all PCR assays should include internal amplification control (IAC) which is typically co-amplified along with the target amplicon (Hoorfar et al., 2004). IAC is to be applied specifically in the amplification step to verify whether amplification reactions have functioned correctly, and identify those which have failed (D'Agostino et al., 2011). Although, an IAC is nucleic acid sequence present in every reaction, which can be co-amplified with the target sequence, it is distinguishable from the target sequence in conventional nucleic acid amplification by band size, and in real-time amplification by different melting temperatures of the amplicons or through detection by IAC-specific probe.
The aim of this paper was to reexamine and determine stability, suitability and significance of previously constructed IACs (Radin and D'Souza, 2011a) for use with earlier published specific COG primers (Kageyama et al., 2003) for the detection of human NoVs genogroups GI and GII from fresh produce and RTE items by real-time RT-PCR assay based on SYBR Green-I chemistry. Here we were interested to investigate the influence of possible contamination of real-time RT-PCR NoV detection assays with inhibitors. These inhibitors could be carried over from RNA extraction from artificially contaminated food items (lettuce, green onions, cherry tomatoes, raspberries, and deli meat). The premise in this work was that if the same concentration of IACs were used in all detections of NoVs and if there is no interference from some food compounds, C_t values obtained for IACs should be the same, or very close. This assumes that the PCR is operating at 100% efficiency, which is ideal or theoretical value. Experimental factors such as the length, secondary structure, and GC content of the amplicon can influence efficiency. Therefore, some slight differences could be expected. If there is significant difference for C_t values it has to represent the influence of diverse inhibitory factors from food, nucleic acid extraction procedures, etc. Inhibition in real-time PCR can be measured as the increase in threshold cycle (C_t) relative to an uninhibited control (Bustin et al., 2005).

MATERIAL AND METHODS

Internal amplification controls (IACs)

Previously constructed RNA IACs for COG set of primers (Radin and D'Souza, 2011a) were used in detection of NoVs genogroups GI and GII from the food samples. IACs could be co-amplified under the same conditions and in the same PCR tube, with the same sets of primers as used for target NoV amplification. IACs are the non-target RNA templates of about 155 bp, with the shiga toxin region of *E. coli* O157:H7 flanked by NoV sequences, containing COG primer binding sites. Due to the RNA nature of IACs they can also be used as a positive control for reverse transcription reaction. The optimal amounts of IACs (0.5 fg of RNA / approximately 5.750 copies of RNA molecules per reaction) were used in real-time RT-PCR assay for the detection of human NoV from food (Radin and D'Souza, 2011a). The optimal concentration considered to be the one that did not decrease the detection sensitivity of the assay (detection of the target product), in the presence of the visible IAC product.

Viral inoculation onto food

All food items (fresh produce: lettuce, green onion, cherry tomatoes; frozen raspberry and sliced fat free turkey breast) obtained from local retail stores were washed with sterile DDI H_2O and 5% tri-sodium phosphate (TSP) prior to inoculation. Additionally, deli meat was kept

under UV light for 3 min prior to inoculation. Food samples (25 g) were artificially contaminated with 100 µl of tenfold serial dilutions of the stool samples (in phosphate-buffer) containing approximately 10^5 and 10^4 RT-PCR units per ml for NoV GI and GII, respectively. After an incubation period of 10 min at room temperature (RT), the food items were processed for viral RNA extraction. Experiment included the samples that were spiked with phosphate-buffer to serve as a negative control during the entire viral detection procedures. All procedures were repeated four times.

Viral RNA extraction

Viral RNA extraction was performed directly from all food items; except from raspberry for which elution/concentration and polyethylene glycol (PEG) precipitation method was used (Baert et al., 2008, Stals et al., 2012). Briefly, treated and untreated food samples were rinsed with 1 ml of TRIzol™ reagent (Invitrogen, Carlsbad, CA). The viral RNA-containing aqueous layer was extracted after the addition of 0.2 ml of chloroform, incubation for 3 min at RT, and centrifugation for 15 min at 10.000 × g and 4 °C. The viral RNA was precipitated in 0.5 ml of isopropanol at RT for 10 min, after centrifugation for 10 min at 10.000 × g and 4 °C. The resulting RNA pellet was washed with 70% ethanol and centrifuged for 5 min at 7.500 × g and 4 °C, air dried, and resuspended in 40 µl of RNase-free water. Following heat treatment at 55 °C an additional purification was done using QIAShredder Mini Spin Column (QIAGEN, Valencia, CA). Extracted RNA was stored at -80 °C. To determine nucleic acid purity optical density measurements were made using Nano Drop® ND-1000 spectrophotometer. Samples of extracted RNA were analyzed by real-time RT-PCR with previously published NoV highly specific degenerate primers COG1F-COG1R (COG1F: 5' CGY TGG ATG CGN TTY CAT GA 3' COG1R: 5' CTT AGA CGC CAT CAT CAT TYA C 3') and COG2F-COG2R (COG2F: 5' CAR GAR BCN ATG TTY AGR TGG ATG AG 3' COG2R: 5' TCG ACG CCA TCT TCA TTC ACA 3') (Kageyama et al. 2003). Primers were obtained from Sigma-GenoSys (St. Louis, MO).

Real-time RT-PCR

Invitrogen SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit was used according to manufactures instruction. Separate mixes for detection of GI and GII were prepared. To chilled PCR tubes 22 µl of cold reaction mix was added followed by 0.5 µl of appropriate IAC and 2.5 µl of the serially diluted extracted viral RNA. The tubes were fast spin and immediately placed into a Bio-Rad iCycler iQ (Hercules, CA). Protocol for COG primers was as follows: reverse transcription at 50 °C/60 min, denaturation at 94 °C/3 min, followed by 50 × cycles at 94 °C/45 s, annealing at 56 °C/50 s and extension at 58/50 s and final extension at 72 °C/7 min. Post-amplification melt temperatures (*Tm*) were obtained after completion of the PCR by plotting the fluorescence intensity against temperature as the temperature was increased from 50 to 95 °C increasing set point after each cycle by 0.5 °C. The threshold cycle (C_t - the cycle number at which the fluorescent signal of the reaction crosses the threshold) was determined by the software. For confirmation of amplicon presence and purity, the real-time RT-PCR product was run on a 2% agarose gel in 1× TAE buffer. A 100 bp DNA molecular weight marker (Promega, Madison, WI) was included to determine size of the RT-PCR product.

Statistics

For comparisons of C_t values Student's T-test from Microsoft Excel was used.

RESULTS AND DISCUSSION

The objective of this research was to assess the influence of the some food types (lettuce, green onion, cherry tomatoes, frozen raspberry, sliced fat free turkey breast) as a possible source for PCR inhibitors. Influence of these inhibitors was determined by addition of IACs to all PCR reactions for amplification of NoV RNA extracted from contaminated food items. This

influence was evaluated as increase in threshold cycle (C_t) relative to an uninhibited control (water). Only PCR reactions positive for IACs but not for NoV RNA, were considered in this study. Mean values for threshold cycle for each food product with respective standard deviation (SD) obtained solely for IACs throughout all the experiments are shown in Table 1.

Table 1.Threshold cycle (C_t) obtained for IACs in the real-time RT-PCR assays based on SYBRGreen-I chemistry in detection of human noriviruses GI and GII with COG primers

Food	Norovirus GI		Norovirus GII	
	Average C_t	SD	Average C_t	SD
Lettuce	25.3	1.3	25.3	1.5
Green onion	28.5	0.4	26.3	0.1
Cherry tomatoes	27.2	1.2	25.9	0.8
Raspberry	25.8	1.4	26.0	1.4
Deli meat	26.6	0.9	25.6	1.3
Control - water	25.9	0.6	25.8	0.7

Inhibition can be assessed by comparing the C_t of the control reaction to which RNAse/DNase-free water had been added with the C_t of the reaction to which NoVs RNA extracted from food had been added. Comparison of C_t values was done with Student's T-test and $P<0.05$ was reconsidered as statistically significant. Reaction to which NoVs RNA extracted from different food showed statistically insignificant difference in values for IACs C_t compared to control. In the case of green onion both for NoVs GI and GII genogroups statistical analysis was not performed as a limited number of data points was available.
After each real-time RT-PCR all reaction products have been run on the agarose gel and the size of IACs amplicons was determined. In Figure 1 it can be seen that in all PCR reaction amplicons of the appropriate size (155 bp) were obtained.

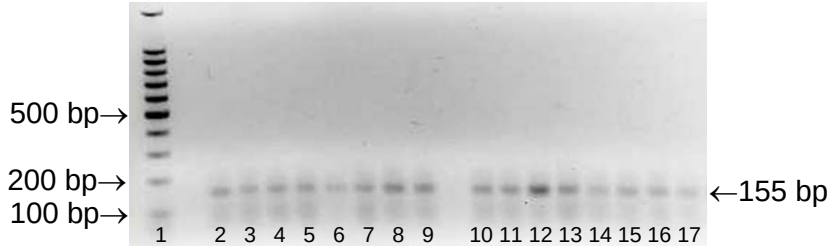

Figure 1. Analysis of IAC real-time RT-PCR amplicon size by gel electrophoresis. *Lane 1* contains 100 bp marker, *lanes 2-6* (NoV GI) and *lanes 10-14* (NoV GII) showing the expected size of 155 bp IACs amplicon in detection of NoV GI and GII RNA extracted from lettuce, green onion, cherry tomatoes, raspberry and deli meat, respectively. *Lanes 7-9* and *lanes 15-17* represent controls, IACs in water.

It was expected that RNA extraction from tested food matrixes would lead to carryover of different compounds and inhibitors for PCR reactions. This would cause PCR reaction inhibitions, which could be observed ether as total PCR failure or increased C_t values for IACs. Surprisingly, we observed no statistically significant change of C_t values for IACs, indicating that PCR reactions weren't affected by the starting material for viral RNA extraction. This could mean that used protocols were effective in reducing the carryover of inhibitory compounds, as seen by unchanged C_t values for IACs. Our results have clearly showed that use of IACs as PCR control is justified. Therefore, IACs provides an accurate way to assess the integrity of all the steps in nucleic acid amplification assays (Nolte, 2004). In general, controls provide evidence that a laboratory assay is meaningful within the environmental system studied (Julian and Schwab, 2012).
Throughout all the experiments, same equipment, reagents and materials have been used, in order to avoid additional sources of results variation, since it is known that for example, microcentrufuge tubes from different manufactures can contribute to the variation of the

results (Hughes et al., 1994). As well, parameters and condition of real-time RT-PCR assays and primers were identical, because different PCR reactions are not equally susceptible to same inhibition by substances co-purified in nucleic acid extracts (Huggett et al., 2008). Furthermore, phenolic compounds from the sample or carried over from nucleic acid purification procedures can inhibit the reaction by binding to or denaturing the polymerase (Young et al., 1993). Since bovine serum albumin proved to be the most effective in overcoming inhibition (McGregor et al., 1996), it was used in these investigations.
Other researchers have had a similar approach like developed SYBR Green RT-PCR assay to detect human and bovine noroviruses and control for inhibition (Scipioni et al., 2008) or the use of single-stranded oligonucleotides of ~ 120 nucleotides as IAC in real-time PCR assays (Burggraf and Olgemoller, 2004) described for *Mycobacterium tuberculosis* complex, hepatitis B virus, herpes simplex virus, and varicella zoster virus. Use of the IAC did not change the detection limits neither of these, nor of ours assays (Radin and D'Souza, 2010; 2011b).

CONCLUSIONS

It is of vital importance to recognize failed NoV RNA amplification in detection assays as it may lead to a false negative interpretation of the results. An internal amplification control can identify failed reactions. Desirable characteristics of IAC to be used in real-time RT-PCR assay are reliability which is reflected in consistent show of a signal in the absence of a target or in the presence of low target concentrations; performance at concentrations, that won't decrease the detection sensitivity of the assay (detection of the target product). This work has shown that IACs amplification was unaffected by the viral RNA extraction procedure from different food items (lettuce, green onion, cherry tomatoes, raspberry, deli meat), indicating that presented protocols are adequate for use in the detection of HuNoVs.

ACKNOWLEDGEMENTS

The authors acknowledge the support of the TN Agricultural Experiment Station, the Fulbright Program and the Ministry of Education and Science, Republic of Serbia (Project No. III 46009).

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MICROBIAL IMMOBILISATION TREATMENTS OF CATTLE HIDES - A NOVEL APPROACH TO HIDE INTERVENTION STRATEGY

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ABSTRACT: Cattle hide decontamination treatments have been recognized as an effective way to reduce microbial contamination of beef carcasses during slaughter and dressing in abattoirs. A range of related techniques – aimed at killing and/or removing pathogens on hides – have been considered in previously published studies. However, reported microbial reductions achievable were relatively limited: around 2-3 logs on decontaminated hides or about 1 log on resulting dressed carcasses. Consequently, to improve the effectiveness of hide treatments, a new approach – treatment to immobilize microbiota on cattle hide rather than to kill it – has been proposed. An insect-produced, natural, food-grade resin (Shellac) was evaluated as an on-hide microbiota-immobilizing agent in a laboratory models system using a sponge-swabbing microbiological sampling method. On hides spray-treated with a Shellac-in-ethanol solution, recoveries of general microbiota (total viable count of bacteria-TVC, *Enterobacteriaceae* counts and generic *E. coli* counts) were greatly reduced: up to 6.6 $\log_{10}$ CFU/cm^2 reductions. The effects of the Shellac treatment were mainly due to immobilization of bacteria on hair by the resin, and to lesser extent due to bactericidal action of the ethanol. Furthermore, post-slaughter but pre-skinning treatment of hides with Shellac-in-ethanol solution, under practical conditions of small commercial abattoir operation, significantly reduced (up to 1.7 log) levels of general microbiota found on final beef carcasses. Overall, in both laboratory- and abattoir-based experiments, microbial reductions achievable by the Shellac-treatment of hides were superior - on both hides and beef - to those achievable by a control hide treatment using rinse-vacuum with sanitizer. Therefore, the Shellac treatment of hides can be considered as an effective alternative approach to hide decontamination strategies to improve beef safety.
Key words: *cattle hides, hide microbiota, Shellac, microbial immobilisation*

INTRODUCTION

It is well known that the main sources of microbiological contamination of beef carcasses are the alimentary tract and hide of slaughtered cattle. However, in modern abattoirs leakage/spillage of intestinal contents onto the meat occurs rarely, whilst contamination from the hides is a key and inevitable event (Bell 1997, Elder et al., 2000, Vivas Alegre and Buncic 2004, Antic et al., 2010a, Blagojevic et al., 2011). It was found that cattle hide can carry not only up to 11 $\log_{10}$ colony-forming units (cfu)/cm^2 of aerobic bacteria (Antic et al., 2010b), but also some of currently the most important foodborne pathogens, such as *Escherichia coli* O157, *Salmonella* species and *Campylobacter* species, which consequently can contaminate carcass meat (Bell 1997, Elder et al., 2000, Avery et al., 2002, Reid et al., 2002). Therefore, reduction of hide contamination contributes to the reduction of transmission of microorganisms from hide onto carcass and improves the microbial status of dressed carcasses (Nou et al., 2003, Bosilevac et. al., 2005, Antic et al., 2010b). Consequently, studies of various pre-skinning decontamination treatments of hides, aimed at reduction of microbiota levels on hides and subsequent improvements of microbial status of carcasses, have been published (Koohmaraie et al., 2005; Small et al., 2005). The USA regulatory authorities acknowledge that, even with the best hygiene practices, some microbial contamination of the carcass including with foodborne pathogens, is inevitable. Decontamination interventions are therefore perceived as an adjunct to HACCP in meeting pathogen reduction performance standards (Smulders and Greer, 1998). In contrast, until

very recently, the EU regulatory authorities considered that strict hygienic processing should be sufficient to ensure product beef carcass safety, and that introduction of carcass decontamination treatments could carry a risk of producers relying too much on these interventions, so reducing their process hygiene efforts. For carcass decontamination, interventions with substances other than potable water are not used presently although are permissible in the European Union (Regulation (EC) No. 853/2004), but subject to regulatory authorisation and following a risk assessment by the European Food Safety Authority (EFSA).

Published studies on hide decontamination were primarily focused on killing of microbiota on hides, alone or in combination with their physical removal from hides, through use of a range of techniques based on heat (up to 80 ^{o}C) or chemicals (Bosilevac et. al., 2005; Koohmaraie et al., 2005). However, reported microbial reductions achievable under practical conditions in abattoirs were relatively limited: around 2-3 logs on decontaminated hides or around 1 log on resulting dressed carcasses. In principle, to reduce carcass contamination from the hide, it is sufficient to reduce the detachment of bacteria from the hide hair and their subsequent transfer onto the carcass meat - so to kill the bacteria on the hide is not a must. Accordingly, a novel alternative approach to improve the effectiveness of hide treatments in reducing microbial transfer from hides on carcasses has been proposed recently: treatment of hides with Shellac, an on-hide microbiota-immobilizing, natural, food-grade resin (Antic et al., 2010b; Antic et al., 2011).

MATERIAL AND METHODS

Comparison of the Shellac treatment with a hide decontamination treatment in a laboratory model

Shellac (Zinsser Co. Inc., USA) is a food-grade, insect-produced natural resin widely used as a glazing agent in the food (e.g. confectionary and fruit) industry. Pieces of clean and dry hides were treated with the Shellac solution (23% in 99.09% ethanol) by uniform spraying using a commercial sprayer. To evaluate the overall effects of the Shellac hide treatment (based on microbial immobilisation) not only through comparison with untreated hide control, but also through comparison with the effects of a “comparative treatment” (based on killing-removing of microbiota), some of the hide pieces were subjected to decontamination treatment with a sanitizer using rinse-vacuum technique. The sanitizer comprised quaternary ammonium-based surface disinfectant preparation (Fink-Antisept G, FINKTEC GmbH). The preparation was dissolved in warm water (50 °C) and filled into a rinse-vacuum spray extraction cleaning machine (Puzzi 200, Karcher). The hide pieces were treated with the machine in the hair growth direction and subsequently left to dry. Hides were sampled using swabbing technique, because it reflects the way of transfer of bacteria from hides onto carcass meat. After sampling, swabs were processed in laboratory using standard microbiological techniques and Total Viable Count of aerobic bacteria, count of *Enterobacteriaceae* and generic *E. coli* count determined on Petrifilms. Colony counts (CFU/cm^2 of hide) of target microorganisms were converted to $\log_{10}$ CFU/cm^2 before calculation of mean values, standard deviation values and significance of differences between means (T-test).

Evaluation of the effects of hide treatment with Shellac solution on microbial status of beef carcasses in abattoir

The study was conducted in a small commercial abattoir in Serbia, with a total of 57 cattle randomly selected for the trials. The large majority of animals presented for slaughter at the abattoir were visually categorized as dirty: either cleanliness category 3 (dirty; dry or damp, with significant contamination of dirt or faeces) or category 4 (very dirty; dry or damp, with heavy contamination of dirt or faeces). The Shellac or the sanitizer hide treatments were applied after slaughter and bleeding of animals, but before the start of the skinning operation. Hide of each of 18 slaughtered bovines was uniformly sprayed with the Shellac solution using a hand-held commercial sprayer, over the longitudinal area where initial skin-opening

cuts are routinely made during the skinning operation: along the hock (both hind legs)–rump–perianal region–flank–belly–brisket line. In addition, hide of 18 other slaughtered bovines was rinsed-vacuumed with a solution of sanitizer (quaternary ammonium-based surface disinfectant Fink-Antisept G) using a rinse-vacuum spray extraction cleaning machine (Puzzi 200, Karcher) along hide area identically as with the Shellac treatment. Twenty one slaughtered bovines that were not subjected to any pre-skinning hide treatment served as untreated controls. Skinned carcasses of bovines whose hides had been previously subjected to the Shellac solution- or sanitizer rinse-vacuum treatment, as well as of untreated-hide bovines, were surface swab-sampled immediately on completion of the skinning, and microbiological examinations conducted as described for the laboratory model system experiment. The efficacies of each of the Shellac solution- and the sanitizer rinse-vacuum hide treatment in reducing the hide-to-meat microbial transfer (expressed as microbial reductions achieved) were calculated *via* differences between microbial levels found on skinned carcasses of bovines that were previously hide-treated or hide-untreated.

RESULTS AND DISCUSSION

Remarkable reductions of TVC swab-recoveries (average 6.6 log reduction) from natural (uninoculated) hides were achieved by the 23% Shellac solution treatment (Table 1), that were significantly larger (by 1.7 log/cm^2) than those obtained by the sanitizer rinse-vacuum treatment (4.9 log). Overall, the results from this part of the present study demonstrated that the hide treatment combining microbial killing and immobilisation on hair (i.e. Shellac solution in ethanol) is superior to a hide decontamination treatment involving microbial killing and removal from hair (i.e. sanitizer rinse-vacuum treatment), in terms of reduction of general microbiota swab-recovery from treated hides.

Table 1. Comparison of reductions of microbial recoveries from cattle hides achieved by the Shellac treatment and by the sanitizer-based decontamination

	Rinse-vacuum with sanitizer solution (Antisept G) [a]			Spraying with 23% Shellac in ethanol solution [a]		
Microorganisms	Untreated hide	Treated hide	Microbial reduction [b]	Untreated hide	Treated hide	Microbial reduction [b]
Total count of bacteria (log_{10} CFU/cm^2) [c]	9.9±0.3	5.0±0.3	-4.9±0.4^A	10.8±0.2	4.2±0.7	-6.6±0.8^B
Enterobacteriaceae count (log_{10} CFU/cm^2) [c]	5.9±1.2	2.5±0.7	-3.4±1.1^A	4.8±2.4	0.0	-4.8±2.4^B
Generic *E. coli* count (log_{10} CFU/cm^2) [c]	3.9±0.4	1.2±0.4	-2.8±0.7^B	2.9±0.8	0.0	-2.9±0.8^B

[a] *Six correlated treated and untreated hide samples tested*
[b] *Difference between mean values (± standard deviation) of correlated untreated and treated hide samples. Within each row, the values marked by different capital letters are significantly different; B>A (P<0.05)*
[c] *Mean value ± Standard deviation*

In other part of the study, the Shellac hide treatment was further evaluated in a commercial abattoir, to assess its potential to improve microbial status of carcasses in a real-life situation. In abattoir trials, after the Shellac treatment of hides of slaughtered bovines (pre-skinning), the total viable count of bacteria (TVC), *Enterobacteriaceae* counts (EC) and generic *E. coli* counts (GEC) on corresponding beef carcasses (after skinning) were significantly reduced compared to control beef carcasses from untreated-hide bovines - the reductions were 1.7 log_{10} CFU/cm^2, 1.4 log_{10} CFU/cm^2 and 1.3 log_{10} CFU/cm^2, respectively (Table 2). On the other hand, after the pre-skinning sanitizer treatment of hides, TVC, EC and GEC on the skinned beef carcasses were also significantly reduced compared with

control carcasses from untreated-hide bovines – the reductions were 1.0 $\log_{10}$ CFU/cm^2, 1.3 $\log_{10}$ CFU/cm^2 and 1.2 $\log_{10}$ CFU/cm^2, respectively (Table 2). The on-carcass microbial reducing effect of the Shellac hide treatment was significantly better that of the sanitizer hide treatment in case of TVC levels.

Table 2. Reductions of microbial loads on skinned beef carcasses after the Shellac and the sanitizer treatments of hides in small commercial abattoir

Pre-skinning treatments of hides on slaughtered bovines	Parameters	Microorganisms ($\log_{10}$ CFU/cm^2) [a]		
		Total count of bacteria (TVC) on carcasses after skinning	*Enterobacteriaceae* count (EC) on carcasses after skinning	Generic *E. coli* count (GEC) on carcasses after skinning
Untreated hides (control; n=21)	Microbial levels on control carcasses	4.9±0.8 ^{A}	0.8±0.7 ^{A}	0.7±0.8 ^{A}
Hides treated by the Shellac solution (n=18)	Microbial levels on carcasses	3.2±0.7 ^{C}	-0.6±1.1 ^{B}	-0.6±1.0 ^{B}
	Reductions compared with control carcasses [b]	-1.7 ^{E}	-1.4 ^{D}	-1.3 ^{D}
Hides treated by the sanitizer Antisept G rinse-vacuum (n=18)	Microbial levels on carcasses	3.9±1.1 ^{B}	-0.5±1.0 ^{B}	-0.5±1.1 ^{B}
	Reductions compared with control carcasses [b]	-1.0 ^{D}	-1.3 ^{D}	-1.2 ^{D}

[a] *Mean value ± Standard deviation*
[b] *Differences between microbial levels on carcasses with hide treatment and without hide treatment (control)*
n Number of animals examined
A, B, C, D, E: Within the same column and microorganisms, the values marked by different letters are significantly different; C<B<A, D<E (P<0.05)

CONCLUSIONS

Overall, the present study clearly demonstrated that this novel microbial-immobilisation treatment of hides with Shellac-in-ethanol solution greatly reduced “bacterial transferability” as measured through recoveries of microbiota from the treated hides by swabbing and also markedly reduced microbial loads on beef carcasses after Shellac treatment of hides before skinning.

ACKNOWLEDGEMENTS

The study was funded as a part of the EU-FP6 project “ProSafeBeef” (FOOD-CT2006-36241) and Serbian Ministry of Science project “Odabrane biološke opasnosti za bezbednost/kvalitet hrane animalnog porekla i kontrolne mere od farme do potrošača“ (Selected biological hazards for safety/quality of food of animal origin and control measures from farm to consumer, TR31034).

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THE COMPARISON OF VOC COMPOSITION OF ACACIA HONEY AND ACACIA FLOWERS BY GCXGC-TOF MS

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ABSTRACT: Solid phase microextraction (SPME) followed by comprehensive gas chromatography coupled to time of flight mass spectrometer (GCxGC-TOF-MS) was used to characterise volatile organic compounds present in acacia flowers (*Robinia pseudoacacia)* and acacia honey in order to identify volatile compounds that come to honey directly from acacia plant. Acacia honey as well as acacia flowers were collected in Komarno-Komarom district at Slovak-Hungary border. Up to 500 volatile organic compounds were detected in acacia honey at given signal to noise ratio 200, while only 216 were identified. They belong to various chemical classes (hydrocarbons, alcohols, aldehydes and ketones, terpens, benzene derivatives and compounds containing heteroatom). On the contrary, acacia flower scent was characteristic by presence 680 volatile organic compounds.
Key words: *honey, VOC, CGxGC, comprehensive gas chromatography*

INTRODUCTION

Honey belongs to the one of the oldest known food, dating back to ancient times, where it is mentioned in old Sumerian, Babylonian and Egyptian scared texts and writings. Over history, it becomes valuable product that was used as a currency or a special form of tribute. One can distinguish between monofloral and polyfloral honey. Monofloral honey is usually more appreciated, since it primarily originates from the nectar of one type of flower. Monofloral honeys differ in taste, flavor as well as in color depending on properties of primary nectar sources. Up to these days, various analytical methods have been used for determination of botanical origin of monofloral honeys (Kaškoniene et al., 2010) e.g. analysis of pollen (melissopalynology) (Sabo et al., 2011) or monitoring of various analytical parameters, like sugar content (Kukurova et al., 2008), profile of phenolic compounds and flavonoids (Escrich et al., 2011), conductivity, mineral content, pH and colour (Vanhanen et al., 2011), stable isotopes (Rossmann, 2001) etc., but often a correct determination of honey origin requires a detailed knowledge about its physical properties and chemical composition. In the past decade, it was shown that composition of volatile organic compounds (VOC) has to be considered as a perspective tools in the assessment of honey origin. Honey VOCs represents a complex mixture of various low molecular weight organic compounds. Some of them originate from flowers and another are formed during conversion processes of nectar into honey. Thus, VOC in honey could serve as an indicator of botanical origin. Indeed, many papers published within last 10 years deal with characterization of VOC profile of honeys with different botanical origin with special attention to identify their possible markers (Radovič et al., 2001; Castro-Vazquez et al., 2003; Baronni et al., 2006).
Considering fact, that VOC in honey is a complex mixture of different components containing various chemical functional groups with relatively low molecular weight that are present at low concentration levels, their extraction requires utilization of effective sample treatment methods. There are several ways how to extract VOC from honey in order to reach required sensitivity, recovery and reliability. The most frequently used is solid phase microextraction (SPME). This solventless technique can be easily automated, but requires a bit time

consuming optimization of working conditions like type of SPME fibres, sorption temperature, sorption time and desorption temperature. As was mentioned above, honey is a very complicated matrix that contains sometimes several hundreds of different organic compounds with various chemical properties. Thus an application of GC coupled to the universal and specific detector such as mass spectrometer is prerequisite for precise and accurate determination of many chemical compounds in a single run. In spite of considerable developments in chromatography, even high-resolution separations with optimized selectivity may not be sufficient to handle such complex mixtures as honey undoubtedly is. One possibility how to solve problems of overlapped peaks is use multidimensional chromatographic systems with much higher separation efficiency, such as comprehensive gas chromatography (GCxGC). In GC×GC, the entire sample is separated on two different columns, and no information gained during the first separation is lost during the second one. The final chromatogram, except of identification of compounds based on their mass spectra allows also provisionally classify unknown compounds based on the presence of ordered structures for classes of structurally related compounds. Additionally, comprehensive gas chromatography provides two complete sets of retention data for all constituents of a sample, which yields an additional tool for their identification.
The aim of the study presented in this work was to compare VOC profile of acacia honey and acacia flowers from which honey was made in order to identify volatile compounds that come to honey directly from acacia plant.

MATERIAL AND METHODS

Samples of acacia flowers were obtained from acacia trees near village Marcelova at Slovak-Hungarian border. Similarly, studied honeys were collected in 2010 and originate from the same place as collected flowers. In this work 2 samples of collected flowers and 6 samples of acacia honey in different stages of its processing (2 samples from opened honey combs, two samples from sealed honey combs and two samples of stored honeys) were studied in details.
VOCs from flowers and honey samples were extracted by SPME procedure using Gerstel MPS2 autosampler. A 5 g of honey together with a 0.5 g of NaCl were dissolved in DI water in a 20 ml clear glass vials. In case of flowers, a 10 g of flowers were closed in 20 ml clear glass vials. A sample in vials (in case of honey a stirred solution at 450 rpm) was heated at 60 °C for 30 min. VOCs were extracted by SPME fiber for autosampler coated with PDMS/CAR/DVB (50/30 µm thickness) obtained from Supelco (Bellefonte, PA, USA). The fiber was conditioned prior use by heating in the injection port of GC under the conditions recommended by the manufacturer. The adsorption of VOCs from honey samples on SPME fiber took 30 min at 60 °C. Desorption was performed in GC injector in splitless mode at 220 °C for 2 min.
LECO Pegasus 4D (LECO Corporation, St. Joseph, MI, USA) consisting of an Agilent 6890 gas chromatograph and Gerstel MPS 2 autosampler equipped with a CIS injector, dual-stage thermal modulator and secondary oven connected to a Time-Of-Flight Mass Spectrometer. The VOC from honey samples was injected into GC by desorption in GC injector in splitless mode at 220 °C for 2 min. In case of standards, 1 l of solution was injected directly into CIS injector in split mode 1:50. Stored mass range m/z from 29 to 600 with an acquisition rate of 100 spectra/second was used. Helium was used as a carrier gas in constant flows mode 1.0 ml/min. DB-FFAP (30 m × 0.25 mm I.D. coated with 0.25 µm film thickness of nitroterephthalic acid modified polyethylene glycol phase, Agilent, Agilent J &W Column, Agilent Technologies, Palo Alto, CA, USA) was used as 1D column. This column was coupled to 2D column BPX-50 (1.5 m × 0.1 mm I.D. coated with 0104 µm film thickness of 50%-diphenyl-50%-dimethyl polysilphenylene-siloxane), Supelco, Belefonte, PA, USA). Primary oven was programmed from 40 °C (1 min) to 220 °C (5 min) with 2.0 °C/min rate. The temperature offset between secondary and primary oven was 10 °C, 8 seconds modulation period, hot pulse duration 1.5 second, temperature offset of modulation 30 °C, temperature of transfer line 240 °C, temperature of ion source 230 °C, electron impact

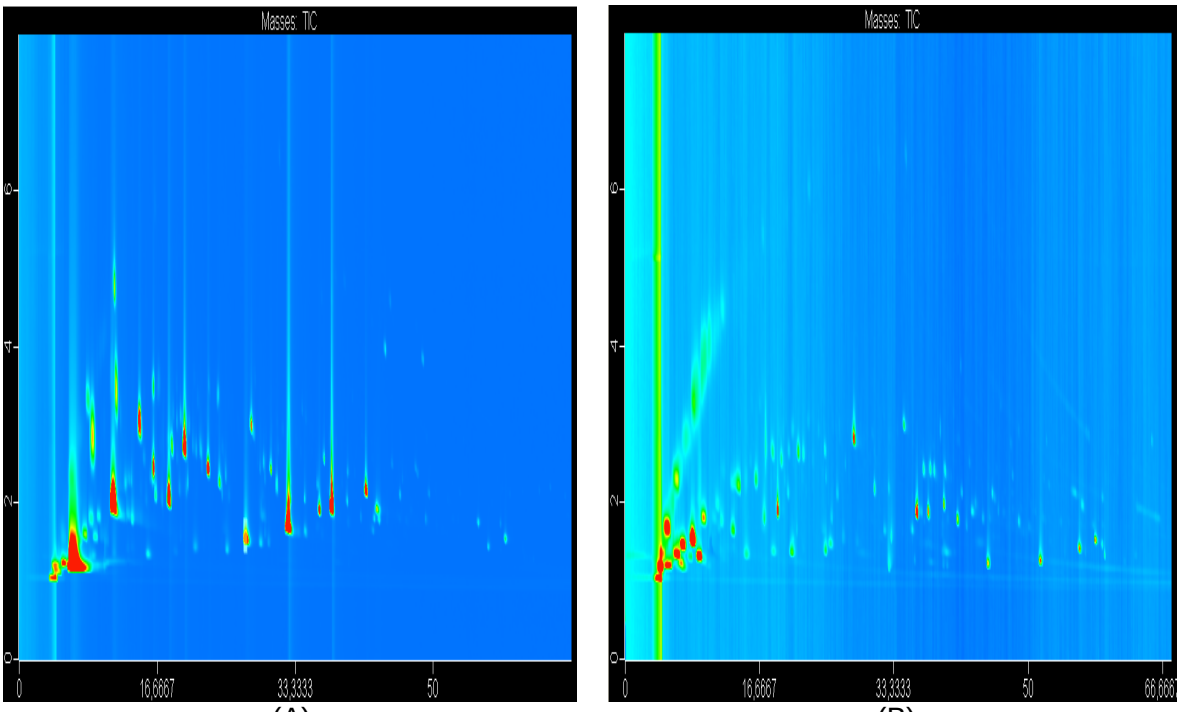
ionization energy 70 eV. LECO ChromaTof software was used for instrument control and data evaluation. Data was processed at S/N ratio 200.
Anhydrous NaCl used in sample treatment procedure was obtained from Mikrochem (Pezinok, Slovakia). DI water was prepared in laboratory using NANOpure device from Wilkem Werner Gmbh. Standard solution of chemicals with concentration 1 ppm was prepared in hexane or methanol (Merck, Darmstad, Germany).

RESULTS AND DISCUSSION

All studied honey samples were analysed under these optimized separation conditions. The chromatograms obtained for acacia flower and honey are shown in Figure 1. The chromatograms were evaluated in order to identify compounds present in honey. The compound was considered as identified only if its retention characteristics in both dimensions, as well as, recorded mass spectra were in agreement with those recorded for standard. If the standard was not available, the identification was performed by comparison of recorded mass spectra with NIST 2008 mass spectra library. However in this case, the compound was consider only as tentatively identified if spectral match higher than 800 was achieved. Homologous series of alkanes starting from pentane up to heptadecane (except of hexadecane), while nonane was present at highest concentration levels, primary, secondary (C4-C10) and tertiary (C5-C8) alcohols, aldehydes (C6-C12), ketonones (except of C4 and C10), organic acids (C1-C16) and their methyl esters have been found in honey.
In total, 509 compounds were found in acacia honey, while only 216 was identified. Benzene derivatives, 2-butanone, alkanes (C6-C9), nonanal, cis-linalool oxide, methyl ester of hexanoic and octanoic acid were present at highest concentration level. Carboxylic acids were found in very low concentration, which could be characteristic for this type of honey. The methyl esters of carboxylic acids were present in relatively high concentration. The presence of all aldehydes from hexanal to decanal is characteristic for this honey. 1-methylpyrrolidinone, 2,4-decadienal (trans isomer), 2-hexen-1-ol, (trans isomer), 3,4-dimethyl-3-hexanol, 3-ethyl-3-heptanol, 3-heptanone, dihydrolinalool were also found in acacia honey. Some aldehydes and terpenes come to honey from primary nectar source, thus those can be used to differentiate honeys based on their botanical origin. A homologous serie of alkanes starting from pentane up to heptadecane (except of hexadecane) with nonane present at highest concentration level were found only in honey samples. On the contrary, VOC profile of acacia flower was characteristic by presence of 2,3-octadiene, 2,4-hetadienal, bicycle [4.2.0] octa-1,3,5-triene, decane, 3-methyl and dodecanoic acid, 2-methyl. Nonen-1-ol, 1-nonen-4-ol, 1-penten-3-ol, 2-octen-1-ol and 2-penten-1-ol were present at highest concentration level. 1,3-octadiene, cyclopentanol, 2,6-octadien-1-ol, 3,7-dimethyl and methyl salicylate were present at lower concentration level.

CONCLUSIONS

In this study, SPME-GCxGC-TOF-MS was used to characterise VOC fraction of acacia honeys in different processing stage as well as corresponding acacia flowers taken from the same geographical area. From all present compounds two important groups were identified. The first group contains compounds that were found either in flowers or only in honey samples. Such example is alkanes identified only in honey samples. Only in acacia flowers were identified example compounds pinene, 4-carene, azulene and eucalyptol. On the other hand, some aldehydes or terpenes come to honey from primary nectar source, thus some of them can be used to differentiate honeys based on their botanical origin.



(A) (B)

Figure 1. The VOC profiles obtained by GCxGC-TOF-MS for acacia flowers (A) and acacia honey (B)

ACKNOWLEDGEMENTS

This work was supported by VEGA grant no. 1/0972/12.

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THE BIOCIDE AND ANTIBIOTIC RESISTANCE IN *Campylobacter jejuni* AND *Campylobacter coli*

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ABSTRACT: The prevalence of antibiotic and biocide resistance among *Campylobacter jejuni* and *Campylobacter coli*, the involvement of active efflux to these resistances and the potential for adaptive resistance after step-wise exposure to increasing sub-inhibitory concentrations of biocides were studied. The antimicrobial resistances were examined with broth microdilution method. The presence of active efflux was studied on the basis of restored sensitivity in the presence of the efflux pump inhibitors (EPIs) and inactivation of the *cmeB, cmeF* and *cmeR* efflux genes. Changes in outer membrane protein profiles (OMPs) and morphological changes were studied in adapted and their parent strains. Among 42 *Campylobacter* strains studied, different antibiotic and biocide resistance levels as well as multidrug resistance were seen, but no correlation between biocide and antibiotic resistance was confirmed. EPIs partially reversed the resistance to all antimicrobials tested. The antimicrobial resistances were reduced in *cmeB* and *cmeF* and increased in the *cmeR* mutant. Repeated exposure to biocides resulted in the partial increased resistance and acquisition of cross-resistance to biocides and antibiotics, which was partially stable. More than one type of active efflux was identified in adapted strains. Different alterations in OMP profiles and morphological changes were also observed. This data suggest that active efflux is a crucial mechanism involved in antibiotic and biocide resistance in *C. jejuni* and *C. coli*. More than one type of efflux is employed. The same is true for adaptation to biocides. However, these adaptations were strains specific; no species specific mechanisms were recognized.
Key words: *Campylobacter, biocide, antibiotic, multidrug resistance, efflux, adaptation*

INTRODUCTION

The thermotolerant *Campylobacter* spp., and especially *C. coli* and *C. jejuni*, have become the most commonly reported bacterial cause of food-borne gastroenteritis in humans worldwide (Moore et al., 2006). Beside this widespread occurrence, they have become increasingly resistant to antibiotics (Engberg et al., 2001).
Biocides are widely used to prevent bacterial contamination in food-processing and in clinical environments (McDonnell and Russell, 1999). In comparison to antibiotic resistance, mechanisms of bacterial resistance to biocides has only been described more recently, and has been less studied overall. A potential concern is the possibility that mechanisms providing resistance to biocides may also provide cross-protection to the activity of antibiotics (Ng et al., 2002). A possible link between biocide and antibiotic resistances in bacteria has been reported in several studies (Copitch et al., 2010; Russel, 2002). However, contrary evidence has appeared in the literature to suggest that this phenomenon does not cause a real problem in practice (Lear et al., 2006; Avrain et al., 2003).
Bacterial resistance to biocides can result from changes in the cell envelope permeability or enhanced biocide efflux (Russell, 2002; Li and Nikaido, 2004). The presence of the efflux pumps CmeABC (Lin et al., 2002; Martinez and Lin, 2006) and CmeDEF (Akiba et al., 2006) belong to the resistance-nodulation cell division family of transporters has been demonstrated to contribute to the antimicrobial resistance in *C. jejuni*. The CmeABC is regulated through the transcriptional repressor CmeR (Lin et al., 2005).
Adaptation to biocides by serial passage in increasing sub-inhibitory concentrations of biocides has been documented for some bacterial species. The development of adaptive

resistance and cross-resistance to dissimilar biocides and increased resistance to antibiotics has occurred after step-wise exposures to biocides (Braoudaki and Hilton, 2005; Tattawasart et al., 1999). Active efflux is one of the mechanisms involved in this resistance (Braoudaki and Hilton, 2005; Thomas et al., 2000). It has also been suggested that alterations in the outer membrane are involved, including for the content of lipopolysaccharide and outer membrane proteins (OMPs) (Tattawasart et al, 2000). This adaptive resistance is also associated with morphological changes (Tattawasart et al, 2000a).
In this study, we examined the prevalence of resistance among 15 *C. jejuni* and 27 *C. coli* isolates from food, animal, human and environmental water sources to five biocides: triclosan (TLN), benzalkonium chloride (BC), cetylpyridinium chloride (CPC), chlorhexidine diacetate (CHA) and trisodium phosphate (TSP), two antibiotics: erythromycin (ERY) and ciprofloxacin (CIP) and sodium dodecyl sulphate (SDS). The involvement of active efflux to these resistances was determined on the basis of restored sensitivity in the presence of the efflux pump inhibitors (EPIs). To investigate the contributions of the CmeABC and CmeDEF efflux pumps to these resistances, *cmeB, cmeF* and *cmeR* mutants of *C. jejuni* strain NCTC 11168 were screened for susceptibility in the presence and absence of EPIs. The potential for adaptive resistance of *C. jeuni* and *C. coli* after step-wise exposure to increasing sub-inhibitory concentrations of these five biocides was investigated to identify the mechanisms underlying resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Fifteen *C. jejuni* and 27 *C. coli* strains isolated from animals (poultry, pig), humans and environmental water were used in this study. Three *C. jejuni* (NCTC1168, ATCC33560, and the K49/4 poultry isolate) and two *C. coli* (ATCC33559, and the 137 poultry isolate) strains were used in the adaptation experiments. The cultures were stored at –80 °C in Brain Heart Infusion broth (Biolife) with 20% horse blood (Oxoid) and 20% glycerol (Kemika). The isolates were cultivated at 42 °C under microaerophilic conditions (3% O_2, 10% CO_2 and 87% N_2) in gas-tight containers on Columbia agar with 5% horse blood (Oxoid).

Mutant strains

Purified DNA from *cmeB* (Lin et al., 2002), *cmeF* (Akiba et al., 2006) and *cmeR* (Lin et al., 2005) mutants (obtained from Prof. Zhang, Iowa State University, USA) was used to transform *C. jejuni* NCTC 11168 using standard biphasic methods for natural transformation and construction of *cmeB, cmeF* and *cmeR* mutants (Wang and Taylor, 1990). The transformants were confirmed by PCR using specific primers as desribed previously (Lin et al., 2005).

Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) of TLN (Merck) BC, CHA, CPC, TSP (Sigma-Aldrich, St. Louis, USA), SDS (Invitrogen), ERY (Sigma-Aldrich) and CIP (Fluka) were determined with broth microdilution method in Muller Hinton broth (MHB, Oxoid) as described previously (Mavri et al., 2012). The MICs were defined as the lowest concentration where no viable cells were present, determined on the basis of fluorescent signals measured using a microplate reader (Tecan) after adding CellTiter-Blue® Reagent (Promega) to the culture media, following the manufacture instructions. The assays were repeated twice in duplicate, to confirm the reproducibility of the MIC data.

Involvement of efflux pump activity

The involvement of active efflux to resistance to five biocides, two antibiotics and SDS were determined using the broth microdilution method as described above in the presence of EPIs phenylalanine-arginine beta-naphthylamide (PAN) (10 mg/ml) and 1-(1-naphthylmethyl)-piperazine (NMP) (60 mg/ml).

Adaptation to biocides
Adapted strains were prepared by step-wise exposure to gradually increasing concentrations of biocides over 15 days. Overnight bacterial cultures (100 l) in MHB were inoculated into fresh MHB containing sub-lethal concentrations of the biocides and incubated for 24 h at 42 °C under microaerophilic conditions. One millilitre aliquots of the 24 h bacterial cultures from the samples with the highest biocide concentrations that permitting growth were transferred into a second series of biocide concentrations in MHB, and incubated as before. This step was repeated every 24 h for a total of 15 passages. The concentrations of the biocides were subsequently increased and ranged as follows: TLN from 1 to 3 mg/l; BC and CHA from 0.063 to 0.35 mg/l; CPC from 0.125 to 0.5 mg/l; and TSP from 2 to 5 g/l, depending upon the growth of the adapted microorganism. The concentrations of the cultures were assessed by measuring the optical density at 600 nm on a UV-visible spectrophotometer (Hewlett Packard) and its purities by streaking them onto selective Karmali agar (Oxoid), after 5, 10 and 15 passages.

Determination of adaptive resistance, cross-resistance and stability of adaptive resistance
The adaptive resistance and cross-resistance were determined for each strain after 5-, 10- and 15-day exposure to gradually increasing concentrations of biocides in MHB. The stability of the adaptive resistance was determined for each adapted strain by subculturing in biocide-free MHB every 24 h, for 10 days. The concentration and purity of cultures were assessed at each stage.

Determination of efflux pump activity: Efflux pump activity was determined for each pre-adapted and adapted strain after 5, 10 and 15 passages on the basis of restored sensitivity in the presence of five EPIs: PAN (20 mg/l), NMP, verapamil, reserpine (100 mg/l) and cyanide 3-chlorophenylhydrazone (CCCP) (0.25 mg/l).

SDS-PAGE analysis of outer membrane proteins (OMPs)
OMPs from adapted strains were isolated after 5, 10 and 15 passages from the cell pellets with ProteoExtract® Transmembrane Protein Extraction kits (Novagen).The concentrations were determined using Protein Assay (Bio-Rad). The absorbance was measured at 595 nm using a microplate reader (Tecan). The proteins were analysed with a NuPAGE® electrophoresis system (Invitrogen) in 12% acrylamide NuPAGE® Novex Bis-Tris gels. The gels were stained with the fluorescent stain ruthenium II tris-bathophenantroline disulfonate (RuBP) (B*iotium)* according to the published protocol (Lamanda et al., 2004) and documented using the G: BOX-HR gel documentation system (Syngene). Protein bands were then quantified with Bionumerics v6.6 software (Applied Maths NV). The concentrations of the sample proteins in the protein bands were calculated as described previously (Zupan et al., 2009).

Transmission electron microscopy (TEM)
The cell morphology was assessed for each pre-adapted and adapted strain after 5, 10 and 15 passages using TEM as described previously (Klančnik et al., 2008). Negatively stained cultures were examined with a CM100 electron microscope (Philips Electronics N.V) with the magnification ranging from 13,500× to 34,000×.

Statistical analysis
Statistical analyses were performed with IBM® SPSS® software v17.0 (T-test and Pearson χ^2 test). Results were considered significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Antimicrobial susceptibility testing

In total, 42 *C. jejuni* and *C. coli* isolates were tested for their susceptibilities to ERY, CIP, five biocides (TLN, BC, CHA, CPC and TSP) and SDS. The range of concentrations for antibiotics and biocides (in two-fold increases) and the breakpoints for antibiotics were used according to CLSI (2007). With regard to antibiotic resistances, 33 out of the 42 strains tested (78.6%) were susceptible and 9 out of 42 (21.4%) were resistant to ERY, and a half of these strains tested (51.4%) were resistant to CIP. The resistance to both antibiotics tested was observed in 16.2% of tested strains. A significant difference in ERY resistance was observed between *C. jejuni* (6.7%) and *C. coli* (29.6%). A higher rate of multi-resistance was also found in *C. coli* (20.8%) than *C. jejuni* (6.7%). Higher rates of ERY resistance among *C. coli* than among *C. jejuni* have been reported previously (Kurinčič et al., 2005). A similar situation was observed for the triclosan resistance. *C. coli* strains were in general more resistant than *C. jejuni* strains. Conversely, the level of BC resistance was higher in *C. jejuni* than in *C. coli*. The overall level of biocide resistance was not significantly different in strains with different antibiotic resistance level. No statistically significant correlation was observed between antibiotic and biocide MICs.

Involvement of efflux pump activity

The efficiencies of EPIs PAN and NMP were determined for each strain, in terms of the resistance to all 8 antimicrobials. Table 1 gives the effects of both EPIs in *C. jejuni* and *C. coli.*

Table 1. The effect of PAN and NMP to MIC reduction in *C. jejuni* and *C. coli*

Species	**PAN** (10 mg/l)			**NMP** (60 mg/l)		
	MIC reduction	**n/N***	**(%)**	**MIC reduction**	**n/N**	**(%)**
Triclosan						
C. jejuni	2 – 128	14/14	(100)	2 – 8	11/14	(78.6)
C. coli	4 – 4000	22/22	(100)	2 – 8	22/22	(100)
BC						
C. jejuni	2 – 128	12/14	(85.7)	2 – 128	13/14	(92.6)
C. coli	2 – 128	22/22	(100)	2 – 128	22/22	(100)
CPC						
C. jejuni	2 – 8	12/14	(85.7)	2 – 8	7/14	(50)
C. coli	2 – 16	21/22	(95.5)	2 – 8	12/22	(54.5)
CHA						
C. jejuni	2	5/14	(35.7)	2	7/14	(50)
C. coli	2 – 16	18/22	(81.8)	2	12/22	(54.5)
TSP						
C. jejuni	2 – 4	3/14	(21.4)	2 – 8	13/14	(92.6)
C. coli	2 – 4	10/22	(45.5)	2 – 8	17/22	(77.3)
SDS						
C. jejuni	2 – 64	13/14	(92.6)	2 – 16	14/14	(100)
C. coli	8 – 256	22/22	(100)	2 – 16	22/22	(100)
ERY						
C. jejuni	2 – 16	14/14	(100)	2 – 16	12/14	(85.7)
C. coli	2 – 64	22/22	(100)	2 – 16	22/22	(100)
CIP						
C. jejuni	2 – 256	5/14	(33.3)	2 – 256	6/14	(42.9)
C. coli	2 – 512	12/20	(60)	2 – 512	8/20	(40)

**The number of strains / the number of tested C. jejun or C. coli strains*

Both of the EPIs can partially reverse the resistances to all antimicrobials tested and expressed different target preferences. PAN was less effective in reversing the susceptibilities in *C. jejuni* than in *C. coli*, which can explain the lower occurrence of antimicrobial resistance among *C. jejuni* than *C. coli.* The effects of the *cmeB, cmeF* and

cmeR gene inactivation on antimicrobial resistance in the *C. jejuni* NCTC 11168 strain and the effects of PAN and NMP addition are given in Table 2. The MICs of the antimicrobials were reduced in *cmeB* and *cmeF* mutants and increased in the *cmeR* mutant, with few exceptions. Both of these putative EPIs further decreased the MICs of the antimicrobials in these mutant strains.

Table 2. The antimicrobial MICs in the absence and presence of PAN (10 mg/l) and NMP (60 mg/l) in C. jejuni NCTC 11168 reference and mutant strains. Changes of at least 4-fold are indicated in bold

Strain	MICs							
	TNL (mg/l)	BC (mg/l)	CPC (mg/l)	CHA (mg/l)	TSP (mg/l)	SDS (mg/l)	ERY (mg/l)	CIP (mg/l)
C. jejuni reference strain								
NCTC 11168	64	1	2	1	8	512	0.125	0.25
+ PAN	16	0.5	1	1	8	512	0.031	0.25
+ NMP	16	0.25	2	1	1	256	0.031	0.25
NCTC11168 *C. jejuni* mutant strains								
cmeB	32	0.25	4	0.5	8	128	0.016	0.063
+ PAN	8	0.031	1	0.125	8	4	0.016	0.016
+ NMP	4	0.125	4	0.5	2	32	0.016	0.016
cmeF	128	0.5	0.5	0.5	16	512	0.5	0.25
+ PAN	64	0.5	0.5	0.5	16	512	0.063	0.063
+ NMP	64	0.25	0.5	0.5	8	512	0.063	0.063
cmeR	128	0.25	2	0.5	16	1024	0.5	0.125
+ PAN	64	0.125	1	0.25	16	1024	0.063	0.063
+ NMP	64	0.25	2	0.5	8	1024	0.25	0.125

Adaptation to biocides

Repeated exposure of *C. jejuni* and *C. coli* to biocides resulted in partially increased resistance and acquisition of cross-resistance to dissimilar biocides and antibiotics (data not shown). The developed resistances were partially stable. Active efflux is one of the mechanisms involved in this adaptive resistance. More than one type of efflux pumps was identified on the basis of restored sensitivity in the presence of five different EPIs that block different types of efflux pump systems (data not shown). According to the noted changes in the OMP profiles and their content in the adapted strains, this could be an additional mechanism involved in adaptation. However, each of the adapted strains had different alterations in their OMP profile. Electron micrographs revealed several morphological changes after adaptation, which differ between the different biocides, as well as between the individual strains.

CONCLUSIONS

Among 42 *Campylobacter* strains studied, different antibiotic and biocide resistance levels were seen. The multidrug resistance was also observed, but no correlation between biocide and antibiotic resistance was confirmed. Active efflux is a crucial mechanism involved in antibiotic and biocide resistance in *C. jejuni* and *C. coli*, wherein more than one type of active efflux is employed. Repeated exposure to biocides resulted in the partial increased resistance and acquisition of cross-resistance to biocides and antibiotics. Developed resistance was partially stable. More than one type of active efflux was identified in adapted strains, which expressed also different alterations in OMP profiles and morphological changes. However, these adaptations were strains specific; no species specific mechanisms were recognized.

ACKNOWLEDGEMENTS

The authors are grateful to the Ministry of Higher Education, Science and Technology of the Republic of Slovenia for financial support for this project, and especially for a PhD grant to A.M. that enabled this study on *Campylobacter* spp.

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SUNFLOWER (*Helianthus annuus* L.) FRUIT FRACTURE RESISTANCE

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ABSTRACT: An objective of this study was to evaluate the behavior of three domestic sunflower hybrid fruits on compressive loading considering seed moisture content. Those were Bačvanin, Krajišnik and Šumadinac hybrids. The mean bio yield force, deformation, work and bio yield modulus are presented, as a result of quasi-static fracture measurements of fruits. A sample of 15 seeds was randomly selected from the bulk of each hybrid at four different seeds moisture content range from 5.9 to 36.8% w. b. The fruits of Šumadinac and Krajišnik demonstrate low values of bio yield force from 29.3 to 45.2 N at low moisture content of fruit (between 3.7 and 5.9% w. b.). Opposite result was measured for Bačvanin hybrids (76.2 N) within the same moisture content of seeds. The machine head displacement has increasing tendency as the seeds moisture content increase for all hybrids. The linear model shows a decreasing trend of bio yield modulus for all hybrids as the moisture content of seeds increased, with high values of the coefficient of regression.
***Key words*:** sunflower, physical properties, compressive loading

INTRODUCTION

The sunflower is the most important oil crop in the Republic of Serbia. According to data from the Statistical Office of the Republic of Serbia (www.webrzs.staserb.sr.gov.rs), over the last decade, cultivable sunflower land comprised approximately 170000 hectares, with the gross production of 380000 tonnes of grain per year. Approximately 92% of this production occurred in Vojvodina Province. Sunflower farming has a long tradition in the Balkan Peninsula. Sunflower fruit is the raw material used for sunflower oil production for human consumption, and its defatted meal, as a feed for livestock, has advantages over other oilseed farm crops.

By botanical definition, the sunflower is a fruit or seed consisting of two main parts. The hull or pericarp is a visible outer component that protects the inner kernel. Information about sunflower seed and kernel physical and mechanical properties are necessary for seeding, harvesting, handling, drying, storage and further processing. The quality of sunflower kernels is not evaluated solely by the physical traits like three perpendicular dimensions, surface area, sphericity, thousand kernels weight, bulk and true density, porosity and static coefficient of friction. The behaviour of the sunflower fruit during compressive loading is one of its textual properties. The processing of sunflower for food and feed requires various types of mechanical treatment that depend on external forces, for instance the compression loading behaviour affects milling process or hullability, therefore is necessary data for every new hybrid. The intensity of the external force necessary to crush the fruit depends on the composition of the kernel and the arrangement of its structural elements. The study of the behaviour of the non-homogeneous organic structure of sunflower kernels on compression loading offers a basis for general conclusions regarding how this type of change might be achieved. Furthermore, because compression behaviour is important in sunflower processing, studies that measure such behaviour over a wide spectrum of kernel moisture content are desirable.

The compression loading test shows the response of biomaterials to an applied external force that deforms the body and induces a change in dimension, shape or volume. This test provides important information about elastic or plastic behaviour. The stress-strain uniaxial compression test shows the response of biomaterials to an applied external force. The

stress-strain curve is a graphical measure of the mechanical properties of a biomaterial. The stress-strain biomaterial behaviour should be differentiated from hardness, which defines the resistance of metal to deformation, usually by indentation. This term may also refer to resistance to stiffness or temper, or resistance to scratching, abrasion, or cutting.

The stress is an external force (F) upon a cross section area (A_o) of the specimen. An important aspect is not the quantity of force, but rather that it is applied to a cross section area (Mohsenin, 1980). This is the reason the specimen is a regular shape such as a cylinder or cube. The stress is identified as compressive, tensile or shearing according to the straining action and it is referred to using the Greek letter σ. The unit of stress is (N m^{-2}) and its equation (Đorđević, 1999) is:

$$\sigma = F/A_o \quad (1)$$

Compressed biomaterial changes in length (L). The ratio between displacement (δ) and initial specimen length (Lo) is strain (ε). The unit of strain is nondimensional (-) and the equation is:

$$\varepsilon = \delta/L_o = (L-L_o)/L_o \quad (2)$$

where displacement is $\delta = (L-L_o)$. The strain is an internal reaction of the biomaterial particles induced by the external force. The stress-strain diagram is a graphical presentation of the values simultaneously recorded for stress and strain. The graph indicates biomaterial properties associated with elastic and inelastic behaviour. The typical shape of this curve is presented in Figure 1. Point P in the figure is known as the proportional limit. Stress and strain are linearly correlated. Therefore, the slope of the straight line section is termed the modulus of elasticity, E, or Young's modulus

$$E = \sigma/\varepsilon \text{ or } \sigma = E\,\varepsilon \quad (3)$$

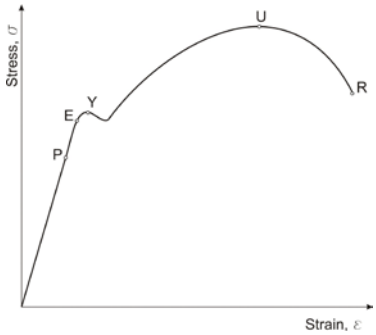

Figure 1. Stress-strain curve

The unit of Young's modulus is (N m^{-2}). The equation (3) is the well-known Hooke's law. The point E in Figure 1 represents the maximum stress that can be applied without resulting in permanent deformation when the specimen is unloaded, whereas point Y is the elastic limit or bio yield point force. After the yield point, the resistance of the material to stress decreases. Therefore, after the peak of the curve, the "yield of displacement" is observed.

Previous studies have described the physical and mechanical properties of sunflower seeds. Jafari et al. (2011) and Santalla and Maschroni (2003) determined sunflower seed properties as a function of moisture content, while Perey et al. (2007) reported similar results for wild sunflower seeds. The compressive load behaviour of sunflower fruits is tested by applying an external force F (N), upon the specimen. The stress-strain diagram represents simultaneous values of force and head displacement (stress) recorded during testing. Within the biomaterials, such as sunflower fruits, the bio yield point force is marked (Mohsenin, 1980). From an engineering point of view, information about the value of the bio yield point force F (N), and head displacement or deformation δ_H (mm), for different hybrids is interesting and relevant. The behaviour of sunflower seeds during compressive loading was reported by Gupta and Das (2000), and the results emphasised the differences of seed orientation (horizontal and vertical) at different seed and kernel moisture contents and the corresponding effects on rapture force, deformation and energy absorbed. The authors concluded that compressive rapture force for unhulled seeds and kernels decreases as the moisture content increases, and higher force values were observed for seeds in the vertical orientation.

Khodabakhian et al. (2011) conducted similar research with three sunflower hybrids, as Voća at al. (2008). Similar to the sunflower, the physical and mechanical properties of other biomaterials are available, like for wheat (Babić et al., 2011; Dobraszczyk et al., 2002), vetch seed (Taser et al., 2005), for soybean kernels (Babić et al., 2001), popcorn seed (Karababa, 2006), chick seed (Konak et al., 2002), barley (Ozturk and Esen, 2008), oats (Gates, 2004) and legume seeds (Kaskowski et al., 1999).
The objectives of this study were to provide new information describing the compression loading behaviour of three domestic sunflower hybrids at different moisture contents. The results of the study were statistically processed using Statistica (www.statsoft.com) version 9.0 and Microsoft Office Excel.

MATERIALS AND METHODS

Seed samples

Three sunflower hybrids from the 2010 harvest season (Krajišnik, Šumadinac and Bačvanin) were tested. The samples (5 kg) were obtained from experimental fields of the Institute of Field and Vegetable Crops, Novi Sad. The seeds were manually cleaned to remove all foreign matter. Each sample was divided into four groups that were adjusted to different moisture contents for testing compression loading. The seeds were kept in sealed plastic bags and stored in a refrigerator at 4 °C. Before each compression test, the sample was removed from the refrigerator and allowed to equilibrate to room temperature. The moisture content was determined according to Regulations about agricultural crops quality (1987) and expressed as Mc (%) on wet basis.

Mechanical properties

Tests of sunflower seed compression in this study were conducted using whole non-grade fruits. Compression tests were applied to fruits in the horizontal orientation. Fifteen replicate tests were conducted for each hybrid, and four different moisture contents of each hybrid. The testing equipment consisted of a loading cell and a computer running the TMS-PRO Texture measurement system (Food Technology Corporation); a trigger load from 0.5 to 450 N was used. The constant deformation rate was 60 mm/min before contact with the specimen and 30 mm/min during compression. The bio yield modulus of elasticity, Ey (N/mm), is the ratio of bio yield force F and machine head displacement or deformation mean value, δ_H (mm), for certain hybrids and moisture contents (Mohsenin, 1980).

RESULTS AND DISCUSSION

The mechanical behaviour of the sunflower fruit under uniaxial loading is a function of its moisture content and genotype. The presence of a larger amount of water in the hull and kernel increases the volume. The water molecules enter the polymeric chain and force it to rearrange, which effects on the compressive behaviour of the whole fruit. The bio yield point force-moisture content curves for three sunflower hybrids are shown in Figure 2. The hybrid Bačvanin attains maximum bio yield forces of 69.7 N with a moisture content of 12.8%. Further increasing the seed moisture content causes a decreasing of bio yield forces. A similar trend was observed by Gupta and Das (2000) during the sunflower seed compression test with horizontal orientation of seed. The same authors emphasised that the hull is fragile at lower seed moisture contents. Therefore, the initiation of its rapture occurs at lower bio yield force values. The results of this study confirm data findings for Šumadinac and Krajišnik hybrids. The low value of bio yield force (34.78 N) was measured with Šumadinac at seed moisture content of 12.5%, while Krajišnik demonstrated the force of 47.6% at 12.7% of seed moisture content. The compressive load behaviour of these two hybrids is quite different as the moisture content increase compare to Bačvanin hybrid. This different behaviour probably results from the histological architecture of the hull.

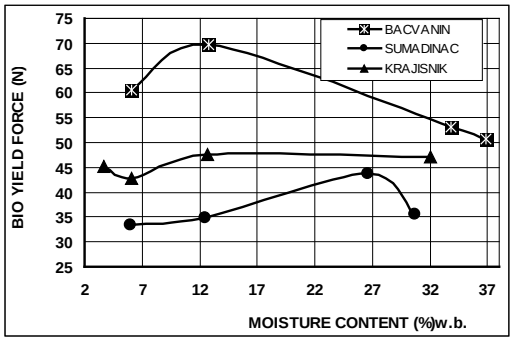

Figure 2. Bio yield force versus seeds moisture content of sunflower hybrids

The two main components of the hull are the sclerenchyma and parenchyma (Hernandez and Belles, 2007), which occupy 20-27% d. b. of the entire fruit. The scerenchyma consists of lignin (20-25% d. b.). The pericarp is arranged in several layers that differ between the cells structure and mechanical properties. The outside of the epidermis is composed of cells that are rectangular in shape. The hypodermis has cells with thin walls. Sclerenchyma cells are polygonal and insert with parenchyma cells at regular spaced intervals in the shape of rays. Such a biochemical construction differs significantly of sunflower genotype and agroecological condition of production, which is the only explanation of the different behaviour. The relationship between bio yield force and the moisture of seed for those hybrids is expressed by the following equations:

For Bačvanin $F=-0.4561Mc+68.742$ $R^2=0.6534$

For Krajišnik $F=-0.0113Mc^2+0.5194\ Mc+42.126$ $R^2=0.5448$

For Šumadinac $F=-0.0066Mc^3+0.3172Mc^2-3.9179Mc+47.016$ $R^2=1$

Deformation (machine head displacement) of the sunflower fruits at bio yield force is plotted in Figure 3. The values of head displacement are a narrow range for all hybrids at lower moisture content because the hull and kernel are both brittle.

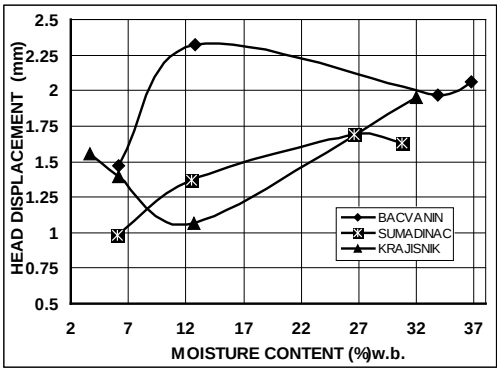

Figure 3. Relationship between head displacement at the bio yield point

The hybrids Šumadinac and Krajišnik expressed an increasing trend of deformation as the seed moisture content increases. The same observation was previously reported by Gupta and Das (2000) and Jafari et al. (2011). The results of this study statistically confirm the linear relationship among the head displacement and seeds moisture content. The equations for these relationships are:

For Šumadinac $\delta_H = 0.025\ Mc + 0.9248$ $R^2=0.8765$

For Krajišnik $\delta_H = 0.0182\ Mc + 1.2426$ $R^2=0.4031$

Bavčanin hybrid exhibited a maximum deformation of 2.43 mm at a seed moisture content of 13.5% w.b. As the seed moisture content of these hybrids increases, the maximum deformation values decrease, according to following equation:

For Bačvanin $\delta_H = -0.0002\ Mc^3 - 0.0175\ Mc^2 - 0.3935\ Mc - 0.3329$ $R^2=1$

The work of bio yield forces at different seed moisture content is presented in Figure 4. The increasing work values occur when the seed moisture content increases (similar to

increasing trends for deformation) for hybrids Šumadinac and Krajišnik. These results are in agreement with the report by Gupta and Das (2000) on absorbed energy of sunflower seed and kernel, for moisture content ranging from 4.21 to 10.65% d. b. Bačvanin hybrid has a decreasing bio yield work tendency as a function of the seed moisture content.

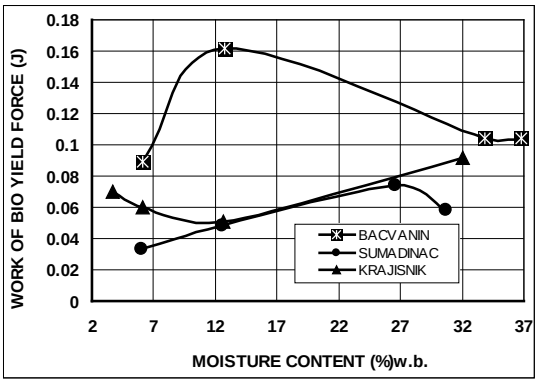

Figure 4. The work of bio yield forces for tested hybrids

Šumadinac, Krajišnik and Bačvanin hybrids demonstrate a decreasing trend of bio yield modulus of elasticity as the fruit moisture content increases. These results statistically confirm the linear relationship between the bio yield modulus of elasticity and the seed moisture content (Figure 5). The equations that express these relationships are:

For Šumadinac	Ey= -0.375 Mc + 33.936	R^2=0.6945
For Krajišnik	Ey= -0.2044 Mc + 30.112	R^2=0.6953
For Bačvanin	Ey= -0.4282 Mc + 40.316	R^2=0.7732

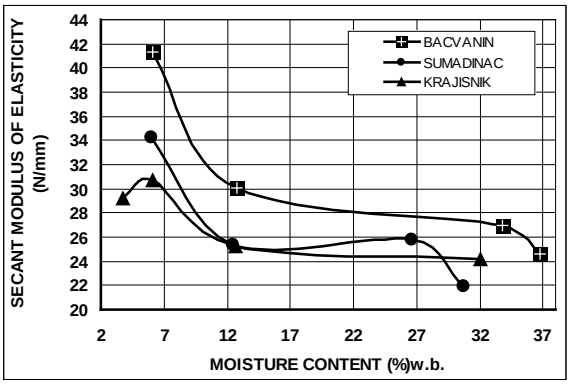

Figure 5. Bio yield modulus of elasticity of tested hybrids versus seed moisture content

The sunflower kernel occupies the inner space only partially, there is intermediate space between kernel and hull filled with spongy and elastic tissue. Those tissues are not a barrier to external force, so after the cracking of the hull, the stress is generated in the kernel. Such stress is not generated in tested hybrids because the hollow inside of the seed is filled with the kernel, which produces some extra resistance to external force. The second important issue that affects the differences in compressive behaviour of these hybrids is the arrangement of pericarp tissues (Hernandez and Belles, 2007), which is characteristic of the genotype.

CONCLUSION

For hybrids Šumadinac and Krajišnik, the increase of bio yield force work values occurs when the seed moisture content increases. The Bačvanin hybrid has a decreasing bio yield work tendency as a function of the seed moisture content. Šumadinac, Krajišnik and

Bačvanin demonstrate a decreasing trend of bio yield modulus of elasticity as the seed moisture content increases.

ACKNOWLEDGEMENT

The study has been granted by Ministry of Education and Science, Republic of Serbia, project No TR-03105.

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PROCESSING QUALITY OF SUGAR BEET, ROOT AND SUGAR YIELD IN RELATION TO CULTIVAR, YEAR AND LOCALITY

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ABSTRACT: Production of sugar beet and granulated sugar in environmental conditions of the Province of Vojvodina is possible but to have a successful production optimal technology must be applied. Production of sugar beet and granulated sugar is virtually impossible without the use of modern cultivars with triple tolerance to the most important diseases: *Cercospora beticola*, rhizomania and *Rhizoctonia solani*. Field microtrials involving cultivars with various levels of tolerance towards the most important diseases of sugar beet were set in 2009, 2010 and 2011, in four replicates. The root yields were determined on the site whereas the chemical analyses were conducted in the laboratory of the Institute of Food Technology in Novi Sad. In 2009, sugar beet showed the best processing quality, followed by those harvested in 2011 and 2010. All seven indicators of processing features were best in sugar beet harvested in 2009. But, the mean root yield was the highest in 2011, reaching the value of 100.02 t/ha whereas it was the lowest in 2009, 88.77 t/ha. The yields of granulated sugar were significantly dependent on cultivar, year and locality. The highest yield of granulated sugar, averaged over cultivars and years, was obtained in 2011 (14.246 t/ha) with Prestige as the best performing cultivar. In contrast, the lowest granulated sugar yield was registered in 2010 with a mean value of 10.566 t/ha, averaged over cultivars and localities. The poorest performance regarding this parameter was shown by cultivar Kontrola I (9.539 t/ha).
Key words: *sugar beet, processing quality, yield, root, granulated sugar*.

INTRODUCTION

The importance of cultivar in the estimation of sugar beet productivity under the agro ecological conditions of the Province of Vojvodina is rather high (Radivojević et al., 1999).
During the last decades, overlapping the period of transitional economy, narrow crop rotation of sugar beet (growing in 2-year rotation or even monoculture) prevailed in large percentage in our country, driven by a desire for quick profit. Hence, it is very important to carefully choose the sugar beet cultivar especially in respect to their tolerance against the main diseases (Märländer, 1991; Uphoff, 2011). The most important diseases occurring under our agro ecological conditions are: *Rhizomania, Rhizoctonia solani*, *Cercospora beticola*, etc.
Taking into consideration the aforementioned, it is important to know the quality of field and level of infestation i.e. the status of inoculums in the field. It is important to note that sugar beet cultivation on certain smaller percentage of fields in the Province of Vojvodina is not considered cost-effective (Radivojević et al., 1999; Radivojević et al., 2008). On these fields, inoculums of different sugar beet diseases have been accumulated causing significantly lower taproot yields with poor processing performances and marginally low granulated sugar yields.
The major growing regions in the Province of Vojvodina differ widely regarding soil fertility and climatic conditions. The four localities included in the field trials are positioned in the regions most favourable for sugar beet cultivation. The years during which the trials were conducted were prominently differing regarding climatic factors.

MATERIAL AND METHODS

Experiments were sown for 3 successive years (2009, 2010 and 2011) and included seven sugar beet cultivars. The trials were settled at four locations across the most important sugar beet growing regions in Vojvodina: Pančevo, Bečej, Sombor and Stara Pazova. Sugar beet genotypes were planted in a randomized block design with four replications at each location and each cultivar was plotted in six rows. The trials were optimally sown in the third decade of March. The microtrial plots were optimally treated regarding fertilization, pest and weed control with respect to conditions characteristic for the growing region. After growing shoots, appropriate weed control was applied including manual weed control which was repeated three times. Besides that, sugar beet crop was protected against *Cercospora beticola*. In 2010, four treatments were applied. Additional fertilization of crops was omitted, only major fertilization as a part of basic cultivation was applied. Taproots were harvested in optimal period, in the middle of October from harvesting plots of 9.80 m^2. The sugar beet cultivars sown in the trials were highly tolerant to *Cercospora beticola* and *Rhisomania*, some of which even to *Rhizoctonia solani*. The studied cultivars are all registered and approved and belonged to the following types: Tibor, Markus, Elmo, Sandor and Elvis were "Z" type whereas Victor and Prestige belonged to „N/Z“ type.
The sugarbeet processing was carried out in the beet laboratory of the Institute for Food Technology in Novi Sad. The analyses of beet composition were performed from fresh beet brei clarified by lead acetate according to the methods published in the Handbook. Sucrose content was determined polarimetrically by a digital automatic saccharometer (Saccharomat IV, Schmidt + Haensch, Germany). Potassium and sodium contents were analyzed by atomic absorption spectrophotometry (Carl Zeiss, Germany). α-amino-N was determined by the Blue number method (spectrophotometer Specord M40, Carl Zeiss, Germany).
The calculation of most important parameters was according to standard formulas (Milić et al., 1992).
The obtained results were analyzed using ANOVA procedure for 3-factorial experiment (Software GenStat v.9., Rothamsted Experimental Station). The factors were cultivars, localities and years.

RESULTS AND DISCUSSION

Taproot yields

Mean taproot yield, depending on cultivar and year was 87.77 t/ha in 2009, 91.43 t/ha in 2010 and 100.10 t/ha in 2011 (Table 1). Maximal variation in taproot yield, averaged over three years was 14.75 t/ha and individual variations by years were significant and ranged from 15.93 t/ha in 2010 to 17.17 t/ha in 2011. Cultivars rang varied due to year among Prestige, Elmo and Victor.
Analysis of variance of the data set showed that the highest influence on taproot yield can be attributed to the interactive effect of year x locality (38.40%), then to the effect of locality (16%) and year (9.69%). Variation due to year was not high which harmonizes with the findings of Webster et al. (1977). The aforementioned effects and their interaction were statistically significant. The influence of cultivar was statistically significant (8.08%) as was the interaction effect between year and cultivar (2.04%). Cultivar-locality interaction was not significant (2.44%). Significant year-cultivar-locality interaction was found (7.76%). The variance of total experimental error was 15.30%.

Table 1. Variations in taproot yield due to cultivar and year

Cultivar	Cultivar type	2009		2010		2011		Mean 2009-2011	
		t/ha	rang	t/ha	rang	t/ha	rang	t/ha	rang
Tibor	Z	87.81	4	91.43	3	98.50	4	92.58	4
Victor	N/Z	83.38	6	91.29	4	103.29	3	92.66	3
Markus	Z	88.83	3	86.31	6	95.21	6	90.11	6
Prestige	N/Z	97.01	1	96.07	2	110.25	1	101.11	1
Elmo	Z	90.26	2	101.78	1	103.50	2	98.51	2
Sandor	Z	86.97	5	87.33	5	96.83	5	90.38	5
Elvis	Z	80.14	7	85.84	7	93.08	7	86.35	7
Annual mean		87.77		91.43		100.10		93.10	
Max deviation		16.87		15.93		17.17		14.75	

LSD (0.05) Year 2.43
Cultivar 3.71
Year x Cultivar 6.42

Sugar content

Mean sugar content in root depending on cultivar and year was 17.59% in 2009, 14.23% in 2010 and 16.56% in 2011 (Table 2). Maximal deviation in the sugar content was 1.02%, averaged over three years and individual deviations by year spanned over the range 1.04% (in 2011)-1.42% (in 2009). Individual variations in this parameter within the cultivars were statistically significant. Over years, cultivars rang varied between cv. Tibor and Markus.

Table 2: Sugar content in root averaged over cultivars and year

Cultivar	Cultivar type	2009		2010		2011		Mean 2009-2011	
		%	rang	%	rang	%	rang	%	rang
Tibor	Z	17.99	3	14.74	1	16.88	2	16.54	1
Victor	N/Z	17.60	4	14.31	4	16.26	5	16.06	5
Markus	Z	18.21	1	14.42	3	16.81	3	16.48	2
Prestige	N/Z	16.79	7	13.61	7	16.14	6	15.51	7
Elmo	Z	16.95	6	13.91	6	16.04	7	15.63	6
Sandor	Z	17.45	5	14.10	5	17.08	1	16.21	4
Elvis	Z	18.13	2	14.56	2	16.71	4	16.47	3
Annual mean		17.59		14.23		16.56		16.13	
Max deviation		1.42		1.13		1.04		1.02	

LSD (0.05) Year 0.10
Cultivar 0.15
Year x Cultivar 0.28

The highest variation in the sugar content was due to the effect of year (51.28%), followed by the interaction of year and locality (32.79%) and locality (7.33%). Similar findings were reported by Glattkowski and Märländer (1994). These effects and their interactions were statistically significant. The influence of cultivar on this parameter was 3.88%. The interaction between year and cultivar was statistically significant. Locality by cultivar interaction was 0.55% and year x cultivar x locality interaction was 1.08%, both of them reaching statistical significance. The variance of total experimental error was 2.23%.

Granulated sugar yield

The yield of granulated sugar content, averaged over cultivars and years, was 13.759 t/ha in 2009, 10.642 t/ha in 2010, 14.389 t/ha in 2011 (Table 3). Average maximal variation in this parameter over the three-year trial was 0.980 t/ha and individual variations ranged from 1.155 t/ha in 2010 to 1.655 t/ha in 2011. Individually, the differences within the tested cultivars were statistically significant. Cultivars rang depended on growing season and varied among Tibor, Prestige and Elmo.

Table 3: Granulated sugar yield averaged over cultivars and years

Cultivar	Cultivar type	2009		2010		2011		Mean 2009-2011	
		t/ha	rang	t/ha	rang	t/ha	rang	t/ha	rang
Tibor	Z	14.302	2	11.304	2	14.552	2	13.386	1
Victor	N/Z	13.070	7	10.846	3	14.472	3	12.796	5
Markus	Z	14.594	1	10.243	5	13.912	6	12.916	4
Prestige	N/Z	14.139	3	10.204	6	15.369	1	13.237	2
Elmo	Z	13.715	4	11.314	1	14.286	5	13.105	3
Sandor	Z	13.410	5	10.159	7	14.420	4	12.663	6
Elvis	Z	13.083	6	10.421	4	13.714	7	12.406	7
Annual mean		13.759		10.642		14.389		12.930	
Max deviation		1.524		1.155		1.655		0.980	

LSD (0.05) Year 0.36
Cultivar 0.55
Year x Cultivar 0.95

According to analysis of variance, major factor affecting the granulated sugar yield was year explaining 34.40% of total variance, followed by year x locality interaction (30.99%) and locality (11.60%). This is in concordance with data reported by Märländer et al., 1994. The influence of cultivar was much lower in relation to the influence of year and locality and their interactions and amounted to 1.28% of total variance. Cultivar by year interaction was also low (2.05%). All these influences were statistically significant. Cultivar by locality interaction was low and did not have statistical significance (accounted for 2% of total variance). But, cultivar x year x locality interaction was 6% and was very significant. The variance of total experimental error was 11.68 %.

Influence of cultivar on the granulated sugar yield was low, accounting to barely 1.28% of total variance. Similar results were reported in several other studies (Märländer, 1991; Wolf and Märländer, 1994; Glattkowski and Märländer, 1995).

Cultivar by year interaction had low impact on the granulated sugar yield (2.05%) but was statistically significant. Much lower influence of cultivar x year interaction on granulated sugar yield was observed by Wolf and Märländer, 1994.

CONCLUSIONS

In this study, the highest influence on the yield of taproots could be attributed to the year by locality interaction (explaining 38.40% of total variance), locality (16.29%) and year (9.69%). The effect of cultivar accounted for 8.08%. The magnitude of other influences was as follows: year by cultivar interaction 2.04%, cultivar by locality 2.44%, and year x cultivar x locality 7.76%. The variance of total experimental error was 15.30%.

Consistently the most important influence on the sugar content in root was exerted by the growing season accounting for 51.28% of total variance, followed by year x locality interaction (32.79%) and locality (7.33%). The effect of cultivar was 3.88%. Other

components of variance were: year x cultivar interaction 0.86%, cultivar x locality interaction 0.55% and year x cultivar x locality interaction 1.08% and total error (2.23%).
The results showed that the granulated sugar yield was most affected by the effect of year (34.40%), locality (11.60%) and cultivar (1.28%). Regarding interactive effects, the highest influence was due to year by locality interaction (30.99%). Other interactions were much less influential. Year by cultivar interaction was 2.05%, cultivar x year x locality interaction was 6% and the lowest was cultivar by locality interaction (2%).

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the Ministry of Science and Education of the Republic of Serbia for the financial support in the frame of Projects 20066 and III 46001.
The authors are also thankful to all farm communities for enabling the settlement of sugar beet micro trials.

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DETERMINATION OF THE AMOUNT OF FREE AMINO GROUPS AS AN INDICATOR OF WHEAT FLOUR PROTEIN COMPLEX QUALITY

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ABSTRACT: The quality of wheat-based products is highly dependent on the quality of the used flour, where the quality of starch and protein fractions has the most dominant role. The degree of protein hydrolysis, which indicates the level of protein quality as well as the end-use flour quality, is determined by different methods (chemical, physical and rheological). The aim of this study was to determine the biochemical status of freshly harvested wheat in terms of the amount of free amino groups as an indicator of the protein hydrolysis degree. Determination of free amino groups was carried out from wet gluten by using modified method of Nielsen at al. (2001), where each sample was beforehand tempered at two different temperatures (30 and 37 °C). The temperature of 30 °C corresponds to the real baking conditions as well as it is commonly used in rheological measurements. Moreover, the temperature of 37 °C provides optimal conditions for the activity of hydrolytic enzymes present. Three wheat varieties were collected from two localities in northern Serbia in 2010/2011 production year. The selected localities were characterized with different micro-climatic conditions during grain filling and maturing. The obtained results indicated that the amount of free amino groups from the samples tempered at 30 °C to a greater extent depends on the locality, whilst the amount of free amino groups from the samples tempered at 37 °C to a greater extent depends on the variety. The further research should be carried out in different stages of post-harvest maturation of wheat as well as in optimal stage of flour maturation.
Key words: *wheat, quality, gluten, free amino groups, micro-climatic conditions*

INTRODUCTION

It is known that protein molecules are linear polymers of amino acids. There are 20 different amino acids, each of them having the combination of a carboxyl group (-COOH) and an amino group (-NH_2) with a side chain that provides the essential difference between all of them. The amino acids are linked together by peptide bonds to form a polypeptide chain (). The sequence and specificity of amino acids are contained exactly in the generic code of the genes, thereby ensuring that the protein will be made in the same manner each time in the specific species and tissue. Amino acids that are considered to be direct products of hydrolysis of proteins are divided into neutral (with equal number of -NH_2 and –COOH groups), basic (with more -NH_2 over –COOH groups) and acidic (with more –COOH over -NH_2 groups) amino acids. The processing properties of a wheat cultivar can significantly vary as a result of climate and cultivation factors (Prieto et al., 1992; Weegels et al., 1988). Recent researches have implicated interference from high temperatures (over 35 °C) during the ripening of the grain. Evidence has been provided that these extreme weather conditions may cause a redirection of protein synthesis, thereby changing the protein quality and thus the dough properties (Wrigley et al., 2004).
The aim of this study was to determine the biochemical status of freshly harvested wheat in terms of the amount of free amino groups as an indicator of the protein hydrolysis degree. The ninhydrin, trinitrobenzenesulforic acid hydrate (TNBS), and fluorescamine reactions and the formol titration technique are commonly used to evaluate the free amino groups. The pH-stat technique is used to evaluate the free α-amino groups (Navarrete del Toro, García-Carreño, 2002).

MATERIAL AND METHODS

Three wheat varieties (Pobeda, Apache and Zvezdana) were collected from two localities in northern Serbia in 2010/2011 production year (Locality 1 and 2).
The localities were selected on the basis of the results of monitoring of microclimatic conditions and the results of commercial and technological quality of the selected wheat varieties during the last decade. Unstable climatic condition during May was suitable for *Fusarium* development, so it was necessary to apply appropriate agro-technical measures. A weather condition in the Locality 1 was characterized by moderate drought, whilst a severe drought was recorded in the Locality 2. Locality 1 was characterized with absolute maximum air temperature ranged from 29 to 30 °C, absolute minimum temperature (1-2 °C), weak and moderate ground frost, as well as short-term poor frost on 2 m height, which did not cause serious damage. Total rainfall ranged from 39 mm (Locality 1) to 46 mm (Locality 2). June 2011 was characterized by variable and moderately warm weather with rainfall deficits. The absolute maximum air temperature in the Locality 1 ranged from 30 to 33 °C, whilst in the Locality 2 ranged from 31 to 34 °C. In July, a period of dry and warm weather allowed the wheat harvest by mid-month to an end. The absolute maximum air temperature in the first week of July stood at 36 °C in the Locality 1 and 38 °C in the Locality 2 (http://www.hidmet.gov.rs).
Wet gluten and protein contents of wheat were determined using near-infrared grain analyzer Infratec 1241 (Foss Analytical AB, Denmark), Besatz content and structure were determined according to Pravilnik o kvalitetu poljoprivrednih proizvoda koji se skladište u javnom skladištu (2010). Wheat samples were milled to flours using laboratory mill MLU 202 (Bühler, Switzerland) (flour extraction rate of 60%). Determination of rheological properties of wheat flour dough was done using the Mixolab (Chopin Technologies, France) according to the standard ICC method 173.

Samples preparation

Wet gluten balls of investigated three wheat varieties were obtained using the method ICC standard No.106/2, and each wet gluten sample was beforehand tempered at two different temperatures (30 °C and 37 °C) during different time intervals (0 min; 90 min; 135 min). The temperature of 30 °C corresponds to the real baking conditions and it is commonly used in rheological measurements. Moreover, the temperature of 37 °C provides optimal conditions for the activity of hydrolytic enzymes present.

Standard preparation

10 mg serine (Art. A0288902) was diluted in 100 ml deionized water to obtain the standard solution, which was used for preparing following dilutions: 0 µg/l; 620.031 µg/l; 1313.625 µg/l; 2774.376 µg/l; 4413.780 µg/l; 6252.855 µg/l; 8333.637 µg/l; 16667.274 µg/l; 33334.548 µg/l; 50001.822 µg/l; 66669.096 µg/l; 83336.37 µg/l and 100003.644 µg/l.

Standard measuring

100 µl serine standard was added to a test tube containing 500 µl o-phtalaldehyde (OPA) reagent and mixed for 5 s. The mixture was left to stand for exactly 2 min and the absorbance was measured at 340 nm (GBC CINTRA 303UV/VIS). This measuring was used to choose the dilution range for calibration curve. Standard calibration curve was constructed each day after setting spectrophotometer at 340 nm.

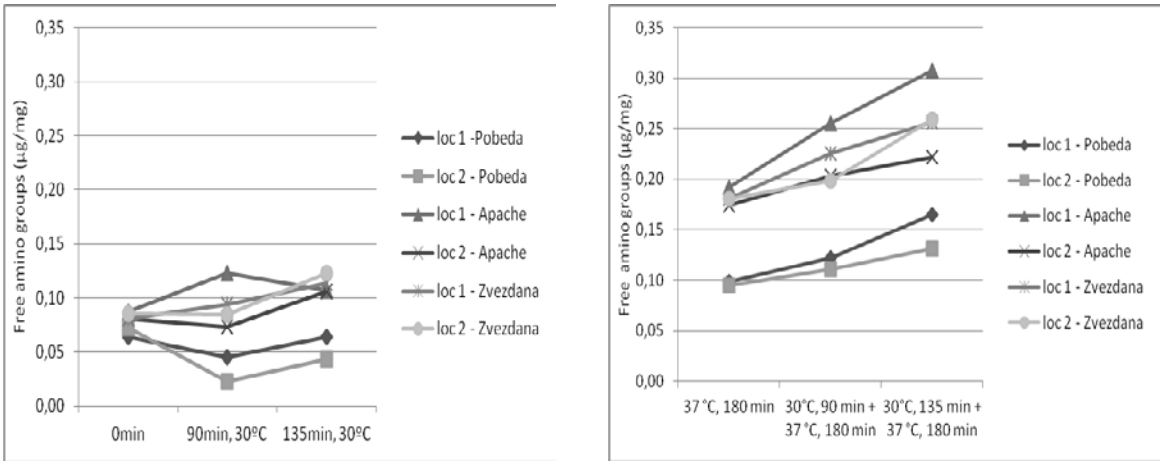
Quantification of amino groups

Changes in amount of free amino groups were determined by spectrophotometric assay using the modified OPA method of Nielsen at al. (2001). All spectrophotometric readings were performed at 340 nm (fixed before measuring with blank reagent (100 µl 1.0 ml KCl solution pH 1.0 and 500 µl OPA reagent containing mercaptoethanol (ME).
Wet gluten (100 mg) was suspended in 1.0 ml KCl solution pH 1.0, vortexed (Vortex V1 plus, BOECO) for 5 min and centrifuged (Centrifuge Minispin + Eppendorf) at 14500 x g for 6 min.

After that, 100 µl of clear supernatant was added to 500 µl OPA reagent containing ME, vortexed for 5 s, and transferred to test tubes (0.7 ml). As absorbance changes somewhat with time, it is important to leave the samples for exactly the same time (2 min) before measuring. Four replicates were made for each determination.

RESULTS AND DISCUSSION

The results obtained after incubation of wet gluten at 30 °C for 2 hours indicated that there were not significant changes in the content of free amino groups (Figure 1).



a) at 30 °C b) 30 °C plus 37 °C

Figure 1. Effect of wheat damage on the concentration of free amino groups of gluten during incubation at a) 30 °C and b) 37 °C and 30 °C plus 37 °C

The content of free amino groups of selected cultivars from two localities was approximately on the same level at 0 minutes, while incubation during 90 and 135 minutes led to differentiation in the content of free amino groups depending on the locality. After incubation of 135 minutes, the content of free amino groups of tested varieties was approximately on the same level as in the 0 minute.

The content of free amino groups in the tested flours (wet gluten) from physiological immature wheat increased with time during incubation at 37 °C, which provided optimal conditions for hydrolytic enzymes activity.

At the incubation temperature of 37 °C, the examined varieties manifested significant differences in the content of free amino groups. The increased content of free amino groups over time was noticed for all samples. Pobeda had the lowest content of free amino groups, with slightly influence of the locality, noticeable after 315 minutes (at 30 °C 135, and then at 37 °C 180 min) of incubation. The varieties Apache and Zvezdana had higher content of free amino groups compared to variety Pobeda. On the basis of free amino groups' content, variety Apache from both localities was characterized by poor technological quality due to the damage occurred during the grain formation. This was confirmed by the shape of the curve, which was in accordance with the results of Pérez et al. (2005).

Table 1 shows the selected indicators of physico-chemical and rheological parameters of the examined wheat varieties from two selected localities.

The highest content of free amino groups and the lowest protein and wet gluten content of variety Apache could be result of higher amount of wheat-bug damaged kernels. The present proteolytic enzymes influenced the release of certain free amino groups which were hidden in a structure of the protein polymer chain (Aja et al., 2004; Pérez et al., 2005). The variety Zvezdana from both localities showed the similar behavior.

Moreover, another possible reason for the increased content of free amino groups could be the influence of maximum temperatures in June from both localities that were very near the critical temperature for the protein synthesis. These influences affected rheological properties

of dough determined by Mixolab, such as the lowest water absorption and the shortest development time of dough.

Table 1. The selected indicators of physico-chemical and rheological characteristics of wheat

		physico-chemical characteristics of wheat						rheological parameters by Mixolab			
locality	varieties	wheat-bug damaged kernels (%)	spoiled and *Fusarium* infested kernels (%)	kernels with black point (%)	protein content (% dmb)	gluten content (%)	protein content in flour (% dmb)	water Absorption (%)	development time C1 (min)	C2 (Nm)	stability time (min)
1	Pobeda	0.40	0.48	0.86	12.7	26.6	11.5	58.3	6.60	0.51	9.38
	Zvezdana	0.42	0.68	0.46	13.2	27.6	12.2	58.4	8.78	0.53	10.63
	Apache	1.00	0.94	0.28	12.4	25.2	11.0	53.3	1.63	0.51	10.53
2	Pobeda	0.30	0.46	6.16	14.0	30.1	12.7	58.5	9.00	0.51	10.18
	Zvezdana	0.41	1.18	2.84	13.2	27.9	12.3	59.3	6.80	0.49	9.77
	Apache	0.70	0.28	2.54	12.6	25.6	10.9	53.3	1.40	0.55	10.90

dmb - dry matter basis

The variety Pobeda exhibited the least pronounced change in the amount of free amino groups, which was confirmed by the shape of the curve that corresponded to the good wheat protein quality. The presence of spoiled, *Fusarium*-damaged and black point kernels could be the additional cause of technological quality deterioration, due to their adverse effect on kernel protein fraction.
It was demonstrated that simply comparison of technological quality indicators of diverse varieties could be inappropriate if they are of different genetic origin, as Apache.

CONCLUSIONS

The obtained results indicated that the amount of free amino groups of the samples incubated at 30 °C to a greater extent depends on the locality, whilst the amount of free amino groups of the samples incubated at 37 °C to a greater extent depends on the variety. The further research should be carried out in different stages of post-harvest maturation of wheat as well as in optimal stage of flour maturation.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 31007 “Vrednovanje kvaliteta i optimizacija prerade pšenice u svetlu klimatskih promena”, financed by the Ministry of Education and Science, Republic of Serbia.

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ANTIFUNGAL EFFECT OF ESSENTIAL OILS ON *Aspergillus westerdijkiae*, *A. ochraceus*, *A. flavus* AND *Penicillium nordicum*

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ABSTRACT: Essential oils are well known antimicrobials that may be used as alternative to synthetic preservatives in foods. The objective of the study was to evaluate the efficacy of different essential oils and their components on fungal growth and mycotoxins production. Minimal inhibitory concentrations (MICs) of essential oils of oregano, thyme, mint, fennel, pine needles and pinecones, and carvacrol, thymol, menthol and anisaldehyde were determined with broth microdilution method for *Aspergillus westerdijkiae*, *A. ochraceus*, *A. flavus* and *Penicillium nordicum*. Thymol, carvacrol and oregano, thyme and menthol essential oils were most efficient antifungals with growth inhibition of all strains already at concentrations 1/4 or even 1/8 of MICs in solid and liquid medium. Among tested fungi only *A. westerdijkiae* produced ochratoxin A, although growth was inhibited when antifungals were added to tested media. The study provides useful information of the potential application of essential oils as very efficient fungal growth inhibitors, but also of the risk of mycotoxin accumulation irrespective to fungal growth inhibition.
Key words: *Aspergillus*, *Penicillium*, *essential oil, antifungal*

INTRODUCTION

Moulds are natural food, raw material and feed contaminants. Moulds are the reason for food spoilage and thus great economics losses worldwide. Next to the moulds potential to cause yield losses and food decay, many species represent a serious health risk for consumers because of their dangerous secondary metabolites–mycotoxins (Fung and Clark, 2004; Kumar et al., 2008). The majority of mycotoxin-producing moulds can be found in genera of *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* which are also the most abundant contaminants of food and feed. The most important mycotoxins in terms of food safety are aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and trichothecenes (García-Cela et al., 2012).
The growth of fungi in foods is inhibited with application of different technological processes and various chemical additives. The consumers demand less use of synthetic preservatives but still they expect food to be free from microbial growth, toxins and other quality deteriorating factors). Additionally, resistance of moulds to chemical additives has led certain fungicides to become ineffective. There is a great need for new antifungal agents with alternative modes of action (Kabak et al., 2006). Research in recent years have shown that different essential oils can be very effective antifungal agents (Cheng et al., 2006; Bajpai Vivek et al., 2008; Kukić et al., 2008; Ahmadi et al., 2010). Effectiveness of essential oils as antifungal agents may, at least partly, be compared with the MIC (minimum inhibitory concentration) values that are in range from some µg/mL to several mg/mL. The comparison of MIC values is problematic because different essential oils are obtained with different procedures, different methods of testing antifungal efficacy, and different types of fungi, different media, and different conditions of cultivation. Determination of MIC values is not sufficient to define whether the production of mycotoxins is reduced or even increased.

Garcia et al. (2011) showed that in some cases, the inhibition of growth with plant extracts also caused reduced mycotoxin production (*Aspergillus westerdijkiae* produced ochratoxin A only in the control samples without addition of plant extracts), but in others it may be that inhibition of growth leads to stimulation of toxin production (ochratoxin A production by *Aspergillus carbonarius* was in presence of plant extracts higher than in the control for 7 and 21 incubation days). Another study showed that MIC of *Caesulia axillaris* Roxb essential oil for growth inhibition for toxigenic strain of *Aspergillus flavus* was higher (1.0 µg/mL) than for the inhibition of aflatoxin B1 production (0.8 µg/mL) (Mishra et al., 2012). Tian et al. (2011) showed that essential oil extracted from the fruits of *Cicuta virosa* L. var. *latisecta* Celak completely inhibited mycelial production of *Aspergillus flavus* at concentration of 5 µL/mL. Mycelial growth was observed at 4 µL/mL, but aflatoxin B1 production was completely inhibited. At lower concentration of essential oil (2 µL/mL) both growth and the aflatoxin B1 production was reduced to about half that of the control.
The aim of our work was to evaluate the effect of selected essential oils and their components on growth of mycotoxigenic moulds and their mycotoxins production.

MATERIALS AND METHODS

Fungal strains, culture media and conditions

The strains *Aspergillus westerdijkiae* ŽMJ26, *A. ochraceus* ŽMJ28, *A. flavus* ŽMJ30 and *Penicillium nordicum* ŽMJ31 were reference strains or isolated and reported previously to produce mycotoxins (Sonjak et al., 2011), They were incubated on Malt Extract agar (MEA) at 25 °C and on Yeast Extract Sucrose agar (YES) at 28 °C from 10 to 14 days for inocula preparation in concentrations of 10^4 and 10^6 spores/mL.

Essential oils, their components and other chemicals

Essential oils of oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.) (Lakić et al., 2012), mint (*Mentha ×piperita* L.), fennel (*Foeniculum vulgare* Mill.), pine needles (*Abies alba* Mill.) and pinecones (*Abies alba* Mill.), and their pure components carvacrol, thymol, menthol and anisaldehyde (Sigma-Aldrich) were used for antifungal testing. Dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany), 96% ethanol, toluene, ethyl acetate, 90% formic acid, chloroform and acetone (all obtained from Kemika, Zagreb, Croatia) were used as solvents while reagent p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich) as indicator of fungal growth by microdilution method.

Minimal inhibitory concentration (MIC) determination by broth microdilution method

For the broth microdilution test 50 µL of spore suspension (10^4 spores/mL) in Tween solution was added to the wells of a sterile 96-well microtitre plate already containing 50 µl of two-fold serially diluted stock solutions of essential oils or pure components in RPMI-1640 medium. Control wells were prepared with RPMI-1640 medium, essential oils only, spore suspension only and DMSO solvent. Plates were put in a microplate shaker (Eppendorf, Hamburg, Germany) for 1 min and then incubated at 25 °C for 48 h for all tested strains except for *A. flavus*, which was grown at 35 °C for 24 h. To indicate metabolic activity the presence of colour was determined after adding 10 µl/well of INT dissolved in distilled water (2 mg/mL) and incubation under appropriate growth conditions in the dark. The MIC value was determined as the lowest concentration of an essential oil or pure component where no colour was observed. All measurements of MICs were repeated twice. According to the MICs, the most efficient oils were selected for further analysis.

Evaluation of the kinetics of growth inhibition

Stock solutions of the selected oils were added in Czapek Yeast Autolysate agar (CYA) to obtain the final concentrations of ½ MIC and ¼ MIC values. Spore suspension (10^6 spores/mL.) was inoculated on solid growth media containing desired concentrations of oils or components. Control samples without oil were also prepared. The growth inhibition was followed by measuring radial growth in 2-4 days intervals at 25 °C. It was also followed by

broth macrodilution test where stock solutions of essential oils were added in YES medium to give final concentration of ½ MIC values obtained in YES medium. 0.5 ml of spore suspension was added in medium in order to obtain final concentration 10^6 spores/mL. After 7, 14 and 21 days of incubation at 28 °C, fungal biomass was gravimetrically measured after chloroform extraction, filtration and drying to constant mass. Growth inhibition rate was calculated from the proportion of dried biomass in the sample containing essential oil comparing to control samples, both prepared in two replicates.

Detection of mycotoxins production by thin layer-chromatography (TLC)
Analysis was performed after 7, 14, 17 and 21 days of culture growth on CYA with silica gel TLC plates (Merck, Darmstadt, Germany) in solvent systems toluene-ethyl acetate, formic acid (5:4:1; TEF) for ochratoxin A (OTA) and chloroform-acetone (9:1; KAC) for aflatoxin B1 (AFB1). Identification of the mycotoxins with agar plug method was performed by comparison of spots from moulds chromatogram with standards (Samson et al., 2000).

RESULTS AND DISCUSSION

Selection of antifungal agents of the basis of MICs
The antifungal effect of 6 essential oils and 4 pure components was examined against mycotoxigenic fungi *A. westerdijkiae*, *A. ochraceus*, *A. flavus* and *P. nordicum.* According to MIC values determined with microdilution method (Table 1), thymol, carvacrol, oregano, thyme and for *P. nordicum* also menthol, were chosen for further analysis as most efficient essential oils. Thymol among the pure components and thyme essential oil showed the lowest MIC values for all tested strains. Obtained MIC values of essential oils were the basis for further testing.

Table 1. Antifungal activity of essential oils and their components expressed as MIC values

	Strain and MIC value			
Antifungal agent	***A. westerdijkiae***	***A. ochraceus***	***A. flavus***	***P. nordicum***
Thymol (mg/ml)	**0.13**	**0.13**	**0.10**	**0.05**
Carvacrol (µl/ml)	**0.20**	**0.39**	**0.78**	**0.07**
Menthol (mg/ml)	0.43	0.75	0.56	**0.06**
Oregano (µl/ml)	**0.78**	**1.17**	**1.56**	**2.34**
Thyme (µl/ml)	**1.56**	**0.39**	**2.34**	**0.29**
Anisaldehyde (µl/ml)	0.78	0.78	2.34	0.39
Minth (µl/ml)	2.34	6.25	4.69	1.09
Fennel (µl/ml)	2.34	2.34	5.73	4.89
Pine needles (µl/ml)	1.25	9.38	1.25	6.25
Pinecones (µl/ml)	1.25	6.25	1.25	6.25

Fungal growth inhibition on solid media
The results of fungal growth on CYA agar plates are shown on Figure 1 (A-F).
Essential oils or their components determined with microdillution method and added in CYA in concentrations ½ or ¼ MICs, delayed or completely inhibited growth of all tested fungal strains. These results are in consistence with other literature data about thymol and carvacrol antifungal activity against different phytopatogenic fungi (Kordali et al., 2008). Inhibitory effect of essential oils or their components was proportional to their concentration, which was shown in the earlier study where essential oils of 12 medicinal plants including thyme, anise, fennel and mint demonstrate inhibitory activity against *Aspergillus* and *Fusarium* fungi (Soliman and Badea, 2002).

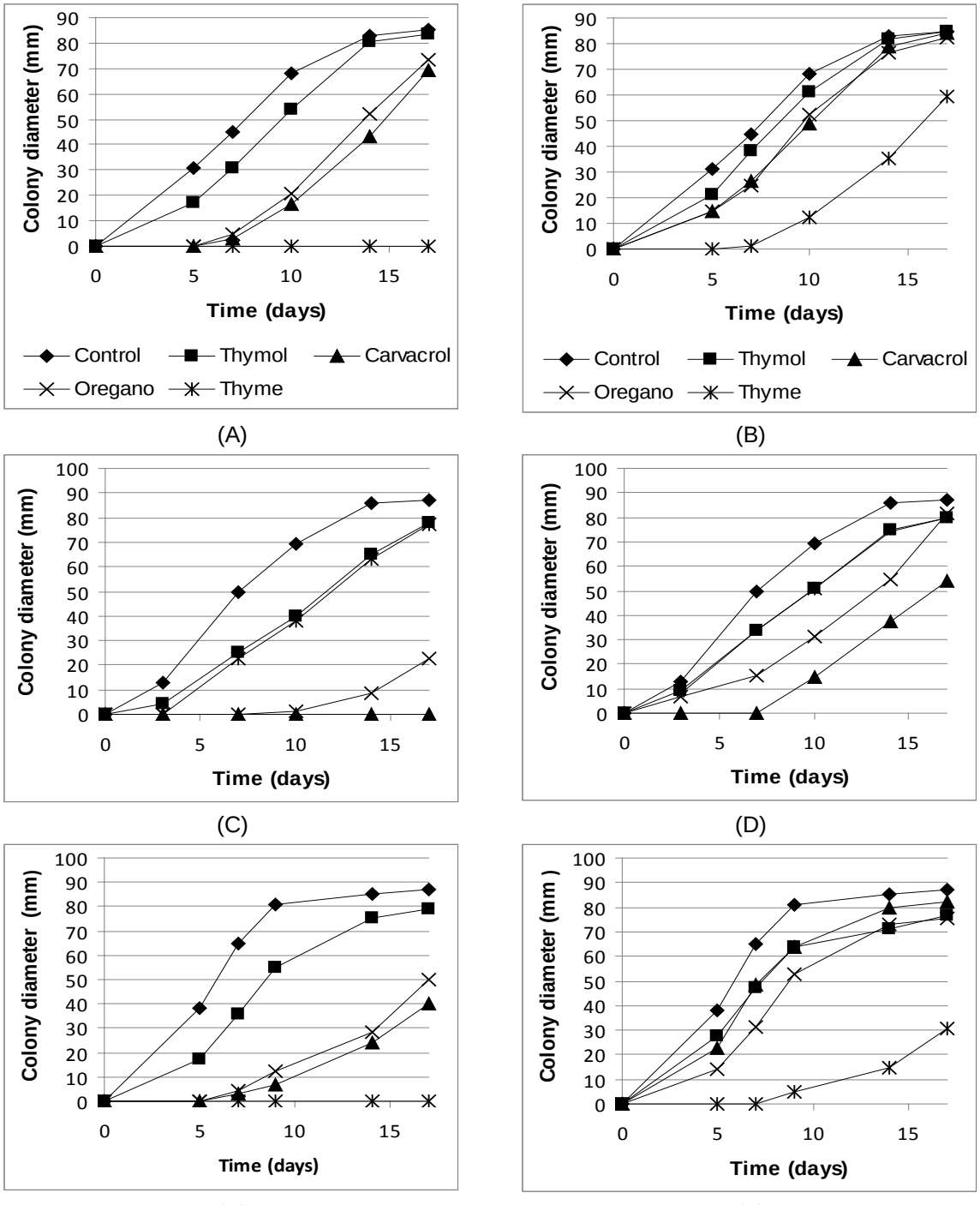

Figure 1. Fungal growth inhibition on CYA with addition of essential oils or their components in concentrations of ½ MIC (left) and ¼ MIC (right) for *A. westerdijkiae* (A, B); *A. ochraceus* (C, D) and *A. flavus* (E, F), respectively.

Fungal growth inhibition in liquid medium:

Thymol and carvacrol as well as thyme and oregano essential oils were the most efficient fungal growth inhibitors on agar plates, so they were tested for their inhibitory effect on *A. westerdijkiae* also in YES liquid medium. Concentrations in range from 1/8 to 1/2 of MICs were tested. These results also indicated antifungal effect of thymol, carvacrol, oregano and thyme essential oils against all tested strains but the efficiency depended on the selected agent and the strain. After 21 days the most efficient agents were oregano essential oil, thymol and carvacrol. Still, there are no reports of essential oils effect against *A. westerdijkiae*, however numerous studies indicate inhibitory effect of thyme, oregano and

thymol essential oils against *A. ochraceus* (Basilico and Basilico, 1999; Soliman and Badeaa, 2002; Nguefack et al., 2009). Until recently these two *Aspergillus* genera were classified as the same *A. ochraceus*, yet however, taxonomical diversity has been recognized (Frisvad et al., 2004). In case *of A. flavus,* growth inhibition in liquid medium was most efficient, including complete inhibition after adding ½ MICs of carvacrol or thyme essential oil.

Detection of mycotoxins production:
The results of determining the formation of mycotoxins by TLC showed that only *A. westerdijkiae* produced OTA in our experimental conditions. When thymol, carvacrol, oregano and thyme in concentrations of ½ MIC and 1/8 of MIC were added to solid and liquid media, the production of OTA was detected by TLC. Quantitative analysis is currently under study to highlight the possibility that mycotoxin production is even induced, when fungal growth is exposed to antifungal components of essential oils and not completely inhibited in growth, as it was reported recently for different *Aspergillus* mycotoxigenic species (Garcia et al., 2011; Mishra et al., 2012).

CONCLUSIONS

The study provides useful information on very effective fungal growth inhibition with plant essential oils and/or their components, especially thymol and thyme essential oils, but also the evidence of the potential risk of mycotoxin accumulation irrespective to fungal growth inhibition. Effective natural alternatives need to be evaluated as the inhibitors of growth and microbial secondary metabolite synthesis, before they can be effectively used in foods.

ACKNOWLEDGEMENTS

The authors are grateful to ARRS for financial support of the project V4-1079 and the Serbian Ministry of Science and Technological Development and ARRS to finance the bilateral project BI-SR-SLO-0034-10/11as well as for CEEPUS grant for M.A. that enabled this study. We are grateful also to Nina Hostnik and Saša Piskernik, who also contributed to this work.

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FATTY ACID PROFILE AND CHEMICAL COMPOSITION OF SOME WARM WATER FISH SPECIES FROM RETAIL STORES

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ABSTRACT: The objective of this study was to assess the chemical and fatty acid composition of representative fish (common carp, silver carp, bighead carp, grass carp, tench, catfish and zander) which were collected from retail stores in area of Novi Sad. Chemical analysis and fatty acid determinations were carried out in the Institute of Meat Hygiene and Technology, Belgrade. The amount of protein was the highest in zander fillets (19.27%) and the lowest percentage of protein was found in grass carp fillets (14.73). Percentage of fat ranged from 1.8, in the muscles of zander, to 10.07 in the meat of carp. The total cholesterol content was the highest in Chinese carps fillets (around 65.38mg/100g), and the lowest in catfish (33.14mg/100g). The amount of saturated fatty acids (SFA) was the lowest in zander (28.6). Tench contained the highest percentage of polyunsaturated fatty acids (PUFA) 34.78% and the lowest percentage was detected in common carp (20.1%). PUFA/SFA, which is an indicator of the quality of lipids was the most favourable in zender 1.17. Also, significant is the ratio of unsaturated (UFA) to saturated (SFA) fatty acids in fish lipids and it was the best in the fat of zender 2.53, but no significance difference among species was not observed (p>0,01). The chemical and fatty acid composition of fish varies greatly between different species and within the same species. Quality of fish meat in our retail stores is quite good but it should be improved by using completed formulated feed mixtures on fish ponds.
Key words*: fresh water fish species, fatty acid, chemical composition, retail stores*

INTRODUCTION

Consumption of fish is increasing based on the recommendations of healthy nutrition (Komprda et al., 2003) and because of the knowledge about fat, protein and cholesterol content, and also quality of fat in fish meat. Ingredients of fish meat are very important in the prevention of cancer (Connor, 2000), brain aging and Alzheimer disease (Kyle, 1999), in the development and sustention of the nervous system, eyes and skin (Lauritzen et al., 2001). Fresh water fish species meat has been assumed to reduce the risk of heart disease, because of low levels of saturated fatty acids (Guler et al., 2008). Further, polyunsaturated fatty acids (PUFA) are often present at high levels in fish meat, especially those of the n-3 series which have particularly beneficial effects on health (Connor and Connor, 2010). The chemical composition of fish varies greatly from one species to another and one individual to another depending on the diet, age, sex, environment and season (Buchtová et al., 2010; Ćirković et al., 2011; Steffens and Wirth, 2007; Celik et al.,. 2005; Guler et al., 2008). Fatty acid composition also varies largely in dependence on the fish species, if they are wild fish or farm-raised, on the age of fish and on origin of diets and its composition (Steffens et al., 2005; Ćirković et al., 2010). Recently, several investigations about meat quality and safety of fish from our fishponds were carried out (Trbović et al., 2009, 2011; Ćirković et al., 2010, 2011, 2011a), but no data is available concerning fatty acids and chemical composition of warm water fish species in our market. The objective of this study was therefore to assess the chemical and fatty acid composition of commercial important fish (common carp, silver

carp, bighead carp, grass carp, tench, catfish and zander) which were collected from retail stores in area of Novi Sad.

MATERIAL AND METHODS

Meat quality of common carp, silver carp, bighead carp, grass carp, tench, catfish and zander which were obtained from retail stores in Novi Sad was analyzed in this study. Eight samples of each fish species were taken from different retail stores and stored at –18ºC prior toanalyses. The meat from dorsal muscles without skin was used for chemical analyses. Chemical composition of fish muscle tissue and feeds was determined by standard SRPS ISO methods. Fatty acids determination was performed according to Spirić et al. (2009) by capillary gas chromatography. Cholesterol determination in carp fillets (from direct saponification) was performed by using HPLC/PDA system (Waters 2695 Separation module/Waters photodiode array detector, USA) on a Phenomenex Luna C18 (2) reverse/phase column, according to Maraschiello et al. (1996). In quantification of cholesterol, external standardization was used, along with Empower Pro software to control the HPLC system for data acquisition and data processing as described (Spirić et al., 2009). Group effect was determined using one-way ANOVA (Statistica 10.0, StatSoft Inc.). Inter-group differences were attained by the Tukey HSD test at $p \leq 0.01$. The results were presented as means ± standard deviation.

RESULTS AND DISCUSSION

Regarding chemical and fatty acid composition of muscle tissue, it was expected to find some major differences within the same species and among different species in percentages of the nutrients monitored between tested fish species because the fish were from the different environment, they were of the different species and age, and were fed with different feed. The results of the chemical composition are shown in Table 1. It was observed that fat content varies the most in common carp (6.3-15%), bighead carp (3-10%), silver carp (4-8%), and the least in zander (1.5-2.2%). Protein content also varied but less than fat content, and variation was also the greatest in common carp (14.1-16.9%) and the lowest in silver carp (17.6-18.6%). Fauconneau et al. (1995) and Romvári et al. (2002) also reported that lipid content of common carp's fillet shows high variance (1–13%) in commercial size of fish depending mainly on previous nutrition. High average values of fat content observed in fillets of Cyprinids, especially in common carp, showed that the energy-protein ratio was not balanced in the feed of these fish in the great number of ponds. Variation within the same species was noted in the amount of total cholesterol, which was the biggest in common carp (43.2-65mg/100g). The total cholesterol content in fillets of carps is in agreement with the data of Wheeler et al., (1987); Vácha and Tvrzická (1995) and Bieniarz et al. (2000), who reported slightly higher cholesterol content in the most analyzed fish species (49-92 mg/100 g) in comparison with meats (45-84 mg/100 g) and it was lower for catfish, zander and tench (33, 42, 45, respectively), which is in agreement with our previous results (Ćirković et al., 2011). The total cholesterol content of the animal tissues can be influenced by the composition of the feed (Komprda, et al., 2003), that could be explanation of variations in cholesterol content because examined fish were from different ponds and fed different feed.
Significant variations in distribution of various fatty acids were noted between and within species. Fatty acid compositions, which are shown in Table 2, were in the wide range especially in fillets of common carp, so C16:0 was in range 17.3-33.4%. The amount of saturated fatty acids was notably constant in all examined species at around 30 %and palmitic acid was the dominant saturated fatty acid and no significance difference between species was not observed (P>0,01). The amount of C18:1c was in wide range in common carp fillets and the least value was observed in silver carp fillets (12.5%). C22:6n-3 varied between species and within the same species, it was the lowest in fillets of common carp and the most favourable in fillets of zander. The greatest deviation of total SFA was observed in

common carp and of content of MUFA and PUFA in catfish and zander. The lowest n-3/n6 ratio was found in one sample of common carp (0.12), and the greatest in one sample of bighead carp (3.7). Obtained results are in agreement with previous reported results referred to studied fish species (Jankowska et al., 2004; Celik et al., 2005; Steffans and Wirth, 2005; Ćirković et al., 2011). All species of analyzed warm water fish contained significant quantities of the n6 series, particularly C18:2 and C20:4. The presence of these and the other polyunsaturated fatty acids emphasizes the potential of freshwater fish for use in special low fat diets.

Table 1. Chemical composition of seven fish species obtained from retail stores

Parameters	Common carp	Silver carp	Bighead carp	Grass carp	Wels catfish	Zander	Tench
Moisture content (%)	73.16±2.81^{a}	74.68±1.49^{a}	74.48±2.44^{a}	76.17±1.67^{ab}	77.74±0.89^{b}	77.89±0.45^{b}	76.13±1.44^{ab}
Protein content (%)	15.64±0.98^{a}	18.01±0.32^{b}	18.03±0.34^{b}	14.73±0.70^{a}	17.34±0.53^{b}	19.27±0.39^{c}	15.71±0.4^{a}
Fat content (%)	10.07±3.18^{a}	6.1±1.59^{bc}	6.29±2.42^{bc}	8.02±1.61^{ac}	3.96±0.69^{bd}	1.8±0.0.23^{d}	6.16±1.14^{bc}
Ash content (%)	1.14±0.09^{ab}	1.21±0.05^{ac}	1.2±0.0.3^{ae}	1.07±0.16^{ad}	0.96±0.12^{d}	1.04±0.02^{bde}	2.00±0.03^{f}
Total cholesterol (mg/100g)	56.38±6.48^{a}	65.38±3.29^{b}	65.2±2.19^{b}	65.29±2.56^{b}	33.14±1.58^{c}	42.34±1.33^{d}	44.97±2.55^{d}

Values are means ± SD (n = 8); Values in the same row with different letter notation statistically significantly differ at p < 0.01/

The reason for the least favourable composition of fatty acid profile in lipids of common carp can be accounted to the type of food dominating in the diet. The traditional approach to the rearing of common carp in the Republic of Serbia is based on foods naturally occurring in ponds (zooplankton, benthos). The energy-producing component of their diet is supplemented with untreated cereals (corn and wheat) (Ćirković et al., 2002). The feed rich in saccharides leads to an increase in the percentage of the oleic acid (C18:1n-9) in body lipids of the fish. At the same time, there is a decrease in the percentage of PUFA n-3 (Fajmonová et al. 2003; Buchtová et al. 2007). According to research conducted by Buchtová et al. (2010) and Ćirković et al. (2010), carp grown on natural food had a high content of both n-6 and n-3 fatty acids and Ćirković et al. (2011) observed that PUFA/SFA ratio was the most favourable in carp fed complete food, and the least in carp fed with maize and wheat. UFA/SFA ratio was also the best in carp fed a complete feed mixture.

Wood et al (2008) have suggested that ratio of PUFA/SFA should be above 0.4 and according that all examined fish species have had favourable (from 0.66 to 1.17) PUFA/SFA ratio. Scollan et al., (2006) have advised that n-6/n-3 ratio should not exceed 4. All studied species meet this suggestion.

The elementary prerequisite for sustainable carp and other fresh water fish production, with favourable chemical and fatty acid compositions should be seen in the development of better feeding procedures. Completed formulated feed mixtures are necessary in modern fish farming because it improves growth performance and chemical and fatty acid composition in fish (Ćirković et al., 2011). Supplemental feeding should contain more vegetable oils that will help to increase PUFA content in muscle lipids of the fish (Steffens and Wirth 2007).

Table 2. Fatty acid composition of seven fish species obtained from retail stores

	Common carp	Silver carp	Bighead carp	Grass carp	Wels catfish	Zander	Tench
Fatty acid (%)							
C12:0	0.24±0.2^{ab}	0.46±0.2^{b}	0.37±0.11^{bc}	0.10±0.04^{a}	0.21±0.14^{ac}	0.2±0.13^{ac}	0.09±0.04^{a}
C14:0	1.47±0.93^{a}	4.49±1.27^{b}	3.82±0.13^{ab}	1.49±0.34^{a}	3.00±1.52^{ab}	2.15±2.41^{a}	1.69±1.2^{a}
C15:0	0.54±0.58^{abc}	1.05±0.23^{b}	0.77±0.39^{abc}	0.29±0.07^{c}	0.70±0.25^{abc}	0.32±0.04^{ac}	0.86±0.16^{ab}
C16:0	22.68±6.07	21.21±2.53	20.2±2.06	22.91±0.37	19.31±2.05	18.23±1.8	22.65±3.27
C16:1	5.93±1.24^{a}	10.04±1.45^{bc}	8.07±1.4^{abe}	10.73±0.01^{c}	9.49±1.57^{bcd}	5.47±1.51^{ae}	7.04±2.1^{ade}
C17:0	1.27±1.64	1.74±0.69	1.17±0.58	0.41±0	1.02±0.46	0.53±0.16	1.09±0.11
C18:0	7.18±2.57^{a}	5.37±1.48^{ab}	6.19±1.68^{a}	3.38±0.12^{b}	5.75±1.02^{ab}	6.98±1.08^{a}	7.34±1.58^{a}
C18:1cis-9	35.84±11.05^{a}	20.55±3.87^{b}	19.84±1.05^{b}	34.37±1.5^{ac}	22.58±4.07^{b}	26.3±8.07^{abc}	17.97±2.31^{b}
C18:1cis-11	2.82±1.81^{a}	4.51±1.1^{ab}	4.16±0.95^{ab}	4.46±0.32^{ab}	6.00±1.85^{b}	4.77±0.42^{ab}	4.79±1.08^{ab}
C18:2 ω-6	9.62±3.48^{ac}	5.13±1.08^{bd}	4.02±1.23^{d}	10.39±2.5^{c}	6.14±1.6^{abd}	9.07±3.23^{abc}	7.33±1.6^{abcd}
C18:3 ω-6	0.45±0.44	0.35±0.25	0.2±0.09	0.11±0.04	0.26±0.23	0.18±0.12	0.17±0.2
C18:3 ω-3	2.71±1.58^{a}	5.91±0.93^{b}	6.27±1.29^{b}	3.75±1.35^{ab}	3.92±1.98^{ab}	2.2±1.52^{a}	3.85±1.2^{ab}
C20:0	0.17±0.07	0.24±0.19	0.28±0.15	0.13±0.05	0.22±0.1	0.21±0.06	0.54±0.18^{b}
C20:1	1.7±0.59^{ab}	1.26±0.2^{a}	1.41±0.4^{a}	1.03±0.06^{a}	3.37±2.29^{b}	1.35±0.51^{a}	1.25±1.23^{a}
C20:2	0.69±0.23	0.41±0.09	0.74±0.37	0.43±0.08	0.65±0.19	0.43±0.25	0.68±0.19
C20:3 ω-6	0.86±0.3^{a}	0.47±0.09^{b}	0.42±0.16^{b}	0.72±0.07^{ab}	0.58±0.21^{ab}	0.50±0.27^{ab}	0.71±0.19^{ab}
C20:3 ω-3	0.64±0.32^{a}	0.67±0.14^{ab}	1.21±0.79^{b}	0.36±0.06^{a}	0.42±0.26^{a}	0.41±0.14^{a}	1.02±0.48^{ab}
C20:4 ω-6	1.85±1.1^{a}	3.4±1.11^{a}	4.05±1.73^{ab}	1.61±0.48^{a}	3.55±2.29^{a}	2.67±1.3^{a}	6.36±1.92^{b}
C20:5 ω-3	1.09±0.69^{a}	5.42±1.52^{bd}	7.84±1.89^{d}	0.96±1.31^{a}	3.43±2.17^{ab}	6.13±1.78^{bd}	4.05±2.25^{ab}
C22:5 ω-3	0.85±0.34^{ad}	1.07±0.2^{abd}	1.14±0.46^{ac}	0.5±0^{a}	1.82±0.68^{bce}	1.55±0.4^{cd}	2.35±0.52^{e}
C22:6 ω-3	1.32±1.04^{a}	6.62±1.7^{b}	7.82±2.34^{b}	1.9±2.5^{a}	7.62±2.94^{b}	10.52±1.74^{b}	8.26±3.46^{b}
SFA	33.55±11.73	34.57±2.74	32.82±2.15	28.72±0.88	30.22±2.78	28.6±2.73	34.27±3.78
MUFA	46.3±11.57^{ab}	36.36±4.58^{bc}	33.48±1.6^{c}	50.6±1.89^{a}	41.43±5.2^{abc}	37.92±9.16^{bc}	31.05±4.52^{c}
PUFA	20.1±6.66^{a}	29.46±4.96^{ab}	33.73±3.36^{b}	20.72±2.9^{a}	28.39±6.97^{ab}	33.67±6.87^{b}	34.78±5.57^{b}
∑ ω -6	13.48±3.76^{ab}	9.78±2.17^{a}	9.31±1.5^{b}	13.26±2.21^{ab}	11.18±1.91^{ab}	12.86±2.75^{ab}	15.25±2.96^{b}
∑ ω -3	6.61±3.22^{a}	19.68±2.95^{b}	24.54±2.87^{b}	7.46±5.11^{a}	17.21±5.34^{b}	20.81±4.34^{b}	19.52±5.1^{b}
ω-3/ω-6	0.48±0.18^{a}	2.04±0.22^{bd}	2.68±0.46^{d}	0.68±0.77^{ac}	1.52±0.32^{b}	1.62±0.17^{b}	1.36±0.6^{bc}
ω-6/ω-3	2.78±2.33^{a}	0.49±0.05^{b}	0.38±0.05^{b}	2.22±0.74^{ac}	0.69±0.16^{bc}	0.62±0.06^{bc}	0.82±0.23^{bc}
PUFA/SFA	0.66±0.29^{a}	0.86±0.21^{ab}	1.04±0.17^{ab}	0.72±0.13^{a}	0.96±0.3^{ab}	1.17±0.17^{b}	1.04±0.28^{ab}
UFA/SFA	2.24±0.87	1.92±0.22	2.06±0.2	2.49±0.12	2.34±0.31	2.53±0.32	1.96±0.37

Values are means ± SD (n = 8); Values in the same row with different letter notation statistically significantly differ at p < 0.01. SFA-saturated fatty acids; MUFA-monounsaturated fatty acids; USFA unsaturated fatty acids; PUFA-polyunsaturated fatty acids from the n-3 (n-3 PUFA) and n-6 (n-6 PUFA) families.

CONCLUSIONS

The meat of warm water fish from our market represents valuable source of healthy nutrition for the consumer. Chemical and fatty acid composition varied between different species and among the same species. The variations were the greatest in common carp which is the most important species in our country and it was probably because different rearing systems on our fish ponds. All examined species have had PUFA/SFA ratio higher than 0.4, andn–6/n–3 ratio was lower than 4 which are the prescribed values recommended from WHO/FAO organization. Components of fish meat, especially common carp can be improved by improvement rearing procedures. Completed formulated feed mixtures which should contain more vegetable oils are necessary in modern fish farming.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TP 31011 “Uticaj kvaliteta komponenata u ishrani ciprinida na kvalitet mesa, gubitke i ekonomičnost proizvodnje (The influence of the quality of the components of food for cyprinid fish species on the quality of meat, losses and the profitability of production)”, financed by the Ministry of Science and Technological Development, Republic of Serbia.

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IRON CONTENTS IN THE LONGISSIMUS DORSI AND SEMIMEMBRANOSUS MUSCLES FOR FIVE PUREBRED PIGS FROM VOJVODINA

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ABSTRACT: The content of iron (Fe) was investigated in *M. longissimus dorsi* and *M. semimembranosus* for five purebred pigs (Large White – LW, n = 6; Landrace – L, n = 6; Duroc – D, n = 6; Hampshire – H, n = 6 and Pietrain – P, n = 6), produced in Vojvodina. Fe was determined by flame atomic absorption spectrometry after mineralization by dry ashing. The difference in the Fe content among the five purebred pigs was not significant in the analysed longissimus dorsi (F = 1.517; P= 0.228) and semimembranosus (F = 0.480; P= 0.750) muscles tissues. Muscles type had no significant effect on the Fe content (F = 2.839; P= 0.097). The order of the purebred pigs regarding Fe content in the longissimus dorsi muscle samples expressed as mg/100g was as follows: LW (1.41–1.81, on average 1.55) > P (1.16–1.77, on average 1.37) > H (1.06–1.62, on average 1.31) > L (1.04–1.65, on average 1.29) > D (1.07–1.57, on average 1.28). The average Fe content in all investigated longissimus dorsi muscle samples was 1.36 mg/100g. The order of the purebred pigs regarding Fe content in the semimembranosus muscle samples expressed as mg/100g was as follows: LW (1.00–2.79, on average 1.57) > P (1.17–1.93, on average 1.52) > D (1.13–1.83, on average 1.47) > L (1.16–1.99, on average 1.46) > H (1.19–1.60, on average 1.37). The average Fe content in all investigated semimembranosus muscle samples was 1.48 mg/100g. The Vojvodian pig meat analysed in this study, showed slightly higher Fe content compared with the values found in other countries.
Key words: *pigs, M. longissimus dorsi, M. semimembranosus, iron*

INTRODUCTION

Meat quality is the sum of all sensoric, nutritive, hygienic-toxicological and technological factors of meat. The nutritive factors of meat quality include proteins and their composition, fats and their composition, vitamins, minerals, utilisation, digestibility and biological value (Olsson and Pickova, 2005; Honikel, 1999; Hofmann, 1990). Red meat (beef, veal, pork and lamb) contains high biological value protein and important micronutrients, including iron, zinc and vitamin B12, all of which are essential for good health throughout life (McAfee et al., 2010; Lombardi-Boccia et al., 2005; Williamson et al., 2005; Higgs, 2000).
The nutrient levels in foods are variable. The major sources of variability in nutrient composition are the wide diversity of soil and climatic conditions (geographical origin), seasonal variations, physiological state and maturity, as well as cultivar and breed (Greenfield and Southgate, 2003). The continuous innovations in the breeding systems, rearing practices, feeds composition, changes in slaughtering methods and ageing, largely contribute to induced changes in the concentration of some micronutrients (Greenfield et al., 2009; Lombardi-Boccia et al., 2005). According to Hermida et al. (2006), the average macrominerals and trace elements concentrations in tissues depend, in part, on the type of cuts, the age of the animals, and various other factors, which are often not reported. Greenfield and Southgate (2003) concluded that the major sources of variation in animal products are the proportion of lean to fat tissue, and the proportion of edible to inedible materials (bone and gristle). Variations in the lean-fat ratio affect the levels of most other nutrients, which are distributed differently in the two fractions.

Dietary sources rich in iron include liver, meat, beans, nuts, dried fruits, poultry, fish, whole grains or enriched cereals, soybean flour and most dark green leafy vegetables. Iron in foods occurs in two main forms: haem and non-haem iron. The major sources of haem iron in the diet are haemoglobin and myoglobin from meat, poultry and fish. Non-haem iron consists mainly of iron salts, derived from plant and dairy products. Most of the non-haem iron present as foods is in the ferric form. Fortification of food with iron is common in developing countries, where deficiency of the element is widespread. In some countries fortification of white and brown flour is mandatory at a level not less than 16.5 mg iron/kg flour. Many breakfast cereals are fortified on a voluntary basis; levels vary but are typically within the range of 70 to 120 mg/kg. Inorganic dietary iron supplements are generally available as ferrous salts (chloride, fumarate, gluconate, glycerophosphate, succinate, sulphate), which are more readily absorbed than ferric salts. Ferrous sulphate and succinate are the most commonly available. Also, water is a potential source of iron (EVM, 2003).

Estimated average daily iron requirements in the UK are 8.7 and 6.7 mg for males aged 11-18 and 19+ years, respectively. For women in the 11-50 years age group the estimated average daily iron requirement is 11.4 mg, whilst that for postmenopausal (50+ years) women is 6.7 mg. Estimated average daily requirements for children are 1.3 mg (0-3 months), 3.3 mg (4-6 months), 6.0 mg (7-12 months), 5.3 mg (1-3 years), 4.7 mg (4-6 years) and 6.7 mg (7-10 years). It has been estimated that the total amounts of iron required for a full gestation is 680 mg. Existing body iron stores should provide this requirement, assuming adequate iron stores at conception, cessation of menstruation and increased intestinal absorption throughout gestation (COMA, 1991). According to FDA (2009), the Recommended Dietary Allowance (RDA) for iron is 18 mg for adults and children four or more years of age.

The majority of functional iron within the body is present in haem proteins, such as haemoglobin, myoglobin and cytochromes, which are involved in oxygen transport or mitochondrial electron transfer. Many other enzymes also contain or require iron for their biological function. Iron deficiency generally develops slowly, and may not be clinically apparent until iron stores are exhausted and the supply of iron to the tissues is compromised, resulting in iron-deficiency anaemia. Groups that are vulnerable to iron deficiency include: infants over 6 months, toddlers, adolescents and pregnant women (due to high requirements); older people and people consuming foods high in iron absorption inhibitors (due to poor absorption); menstruating women or individuals with pathological blood loss (due to high blood losses) (EVM, 2003).

Pig meat is the most widely consumed meat in the EU (Williamson et al., 2005), as well as in Serbia, and the consumption has been steadily increasing. The Autonomous Province of Vojvodina (the northern part of the Republic of Serbia) is a region where the number of animals of the porcine species and the production of pork meat are of high economic importance. Over 30 percent of the total number of pigs slaughtered annually in Serbia comes from Vojvodina. Five purebred pigs (Large White, Landrace, Duroc, Hampshire and Pietrain) and their crosses are used for commercial pork production. In (cross) breeding programme Large White and Landrace are used as female lines and Duroc, Hampshire and Pietrain are used as male lines.

The aims of this study were: (i) to obtain the iron levels of *M. longissimus dorsi* and *M. semimembranosus* from pigs in Vojvodina; (ii) to investigate the potential differences in iron levels of *M. longissimus dorsi* and *M. semimembranosus* among five purebred pigs, used nowadays in Vojvodina for pork production; (iii) to compare the results of our study with results found in other studies, i.e. other countries, for *M. longissimus dorsi* and *M. semimembranosus*; (iv) to calculate the average daily intake of iron through consumption of one serving of 100 g of pork compared to the Recommended Dietary Allowance (RDA). The overall objective is to produce high quality pork to ensure the competitiveness of Vojvodian pork in the international meat markets.

MATERIALS AND METHODS

Animals, sampling and preparing

In this study five purebred pigs (castrates males and females) were used: Large White (LW), n = 6; Landrace (L), n = 6; Duroc (D), n = 6; Hampshire (H), n = 6; and Pietrain (P), n = 6.
The pigs were fattened at the production farms in the northern part of the Republic of Serbia (Autonomous Province of Vojvodina). The pig fattening involved the following phases: starting period (from 15 to 25 kg), growing period (from 25 to 60 kg) and finishing period (from 60 to 110 kg). The diets were based on locally produced corn and soybean meals, and were formulated to meet the nutrient requirements (National Research Council, 1998) for the different growth phases. The finishers were housed in pens with fully slatted floor and 0.80 m^2 space allocation per pig. Each pen contained 10 animals. The environmental temperature in the building was 22°C. All pigs had ad libitum access to a diet and water.
The pigs were randomly selected at an individual live weight between 95 and 110 kg, and were about 6 months old. One pig from each purebred was taken at every six mounts from the same farm. The pigs were slaughtered in the two biggest Vojvodian slaughterhouses according to routine procedure. Carcasses were conventionally chilled for 24 h in a chiller at 2–4°C. After chilling, *M. longissimus dorsi* (LD) and *M. semimembranosus* (SM) were removed from the right hind leg of each carcass. LD and SM muscles were taken from the same animal. The meat samples were trimmed of visible adipose and connective tissue. The samples for chemical analysis (approximately 250 g) taken after the homogenisation of the LD and SM muscle, were vacuum packaged in polyethylene bags and stored at –40°C until analysis.

Analytical methods and quality control

The iron (Fe) content was determined after dry ashing mineralization (Tomović et al., 2011; Gorsuch, 1970). Fe content was measured in the ash solution by flame atomic absorption spectroscopy according to the manufacturer's instructions (Varian Spectra AA 10, Varian Techtron Pty Limited, Mulgvare Victoria, Australia, 1989).
The analysis of the certified reference material (SMRD 2000 – Matrix meat reference material, National Food Administration, Uppsala, Sweden) was used for analytical quality control programme. The results of the analytical quality control programme are presented in Table 1. All analyses were performed in duplicate.

Table 1. The results of the analytical quality control programme (n = 8) used in the determination of the iron in *M. longissimus dorsi* and *M. Semimembranosus*

Element	Fe
Certified concentration (mg/kg)	6.3 ± 3.9
Recovery (%)	101.6 ± 2.51
Limit of detection (mg/100g)	0.50
Limit of quantitation (mg/100g)	0.75

Statistical analysis

All data are presented as average, standard deviation (SD) and range (Min, Max). Independent t-test and analysis of variance (one-way ANOVA) were used to test the hypothesis about differences between two or more average values. The software package STATISTICA (2008) was used for analysis.

RESULTS AND DISCUSSION

Average concentrations, standard deviations and ranges of iron in the *M. longissiumus dorsi* and *M. semimembranosus* tissue samples from five different purebred pigs are presented in Table 2. The minimum Fe content found in all samples was greater than the detection limit.
The order of the purebred pigs according to average iron content in the *M. longissiumus dorsi* samples in mg/100g was: LW (1.41–1.81, on average 1.55) > P (1.16–1.77, on average

1.37) > H (1.06–1.62, on average 1.31) > L (1.04–1.65, on average 1.29) > D (1.07–1.57, on average 1.28). Iron levels found in the present study did not differ significantly (F = 1.517; *P* = 0.228) among *M. longissiumus dorsi* for the different purebred pigs. The average iron content in all investigated longissimus dorsi muscle samples was 1.36 mg/100g.
The order of the purebred pigs according to average iron content in the *M. semimembranosus* samples in mg/100g was: LW (1.00–2.79, on average 1.57) > P (1.17–1.93, on average 1.52) > D (1.13–1.83, on average 1.47) > L (1.16–1.99, on average 1.46) > H (1.19–1.60, on average 1.37). Iron levels found in the present study did not differ significantly (F = 0.480; *P* = 0.750) among *M. semimembranosus* for the different purebred pigs. The average iron content in all investigated semimembranosus muscle samples was 1.48 mg/100g. Muscles had no significant effect on the iron content (*F* = 2.839; *P* = 0.097).

Table 2. Iron levels (mg/100g wet weight) in the *M. longissiumus dorsi* and *M. semimembranosus* of various purebred pigs from Vojvodina

Muscle	Purebred	LW	L	D	H	P	F value	*P* value[1]	All animals
LD	X	1.55	1.29	1.28	1.31	1.37	1.517	0.228	1.36
	Sd	0.14	0.27	0.23	0.19	0.25			0.22
	Min	1.41	1.04	1.07	1.06	1.16			1.04
	Max	1.81	1.65	1.57	1.62	1.77			1.81
SM	X	1.57	1.46	1.47	1.37	1.52	0.480	0.750	1.48
	Sd	0.73	0.30	0.29	0.16	0.27			0.38
	Min	1.00	1.16	1.13	1.19	1.17			1.00
	Max	2.79	1.99	1.83	1.60	1.93			2.79
	F value	0.200	1.033	1.569	0.388	1.014			2.839
	P value[2]	0.666	0.333	0.239	0.547	0.338			0.097

[1]*indicates significant difference between purebred within raw*
[2]*indicates significant difference between liver and kidney tissue within column*

The average iron contents in the *M. longissimus dorsi* and *M. semimembranosus* found in this study were slightly higher compared to the data presented in the food composition tables of other countries (loin: 0.54 mg/100g and ham: 0.73 mg/100g, Australia – Greenfield et al., 2009; loin: 0.71 mg/100g and SM: 0.7 mg/100g, Denmark – National Food Institute, 2009; ham: 0.6 mg/100g, Finland – National Institute for Health and Welfare, 2009; loin: 1.3 mg/100g and ham: 1.6 mg/100g, Italy – INRAN, 2007; loin: 0.7 mg/100g and ham: 0.8 mg/100g, Norway – The Norwegian Food Safety Authority, 2006; loin: 0.84 mg/100g and ham: 1.01 mg/100g, USA – Romans et al., 1994, The US Department of Agriculture's, 2009).

Table 3. Contribution of *M. longissimus dorsi* and *M. semimembranosus* to RDA* for iron in human nutrition (adult and children four or more years of age**; values for the consumption of one serving of 100 g of meat)

Muscle		LD	SM
% RDA (18 mg/day)	Average	7.5	8.2
	Min	5.8	5.6
	Max	10.1	15.5

* *RDA – Recommended* Dietary Allowance
** *Daily values based on a caloric intake of 2.000 calories (FDA, 2009)*

According to obtained results (Table 3), the iron content in 100 g of *M. longissimus dorsi* and *M. semimembranosus* of pigs produced in Vojvodina contributes minimally 5.6% (up to 15.5%) of the RDA value.

CONCLUSION

The results of the present study show that the levels of iron in the *M. longissimus dorsi* and *M. semimembranosus* were not influenced by purebred or muscle. The Vojvodian pig meat showed slightly higher Fe content compared with the values found in other countries.

ACKNOWLEDGEMENTS

These results are part of the project No 114-451-2091/2011 (Improvement of meat quality from indigenous and modern pig breeds produced in Vojvodina for the production of traditional dry fermented sausages and dry cured meat products), which is financially supported by the Provincial Secretariat for Science and Technological Development, Autonomous Province of Vojvodina, Republic of Serbia.

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NOVEL NMR-TECHNOLOGY TO ASSESS FOOD QUALITY AND SAFETY

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ABSTRACT: High Resolution NMR Spectroscopy offers unique screening capabilities for food quality and safety by combining untargeted and targeted screening in one analysis.
The objective of this contribution is to demonstrate, that due to its extreme reproducibility NMR can detect smallest changes in concentrations of many components in a mixture, which is best monitored by statistical evaluation however also delivers reliable quantification results.
The methodology typically uses a 400 MHz high resolution instrument under full automation after minimized sample preparation, which in the case of aqueous solution just needs buffering to adjust pH and in case of cloudy samples centrifugation. For example one fruit juice analysis in a push button operation takes at maximum 15 minutes and delivers a multitude of results, which are automatically summarized in a PDF report.
The method has been proven on fruit juices, where so far unknown frauds could be detected. In addition conventional targeted parameters are obtained in the same analysis. This technology has the advantage that NMR is completely quantitative and concentration calibration only has to be done once for all compounds. Since NMR is so reproducible, it is also transferable between different instruments of the same field strength and different laboratories. Based on strict SOP`s, statistical models developed once can be used on multiple instruments and strategies for compound identification and quantification are applicable as well across labs.
Key words*: High Resolution NMR, Targeted Screening, Quantification, Untargeted Screening*

INTRODUCTION

A new approach, based on ^{1}H-NMR-technology combined with advanced statistical evaluation was developed to supplement conventional analysis (Hofsommer, 1999; Koswig, 2006) with the intention to pre-screen all samples with regard to as many parameters as possible and sort out suspicious ones for confirmation by conventional analysis in order to avoid unnecessarily spent cost for full analysis. The resulting SGF-Profiling (Spin Generated Fingerprint Profiling) for fruit juices (Rinke et al., 2007) can answer this request by using chemo metric comparison of spectrum fingerprints for authenticity control using non-targeted screening procedures. The method is especially successful when used in routine control. It provides additional benefit without creating further costs, delivering targeted analysis of multiple relevant compounds, e.g. indicators for microbiological spoiling or deviations during process or storage. In addition, the structure analysis capability of the NMR technique helps to identify problems caused by unknown compounds. In addition an overview will be presented on further possibilities for food material screening beyond fruit juice. This covers wine, edible oils, cheese and milk powder. It is shown, how the untargeted part of the NMR approach could have immediately resolved the melamine problem, if applied to baby milk powder.

MATERIALS AND METHODS

The SGF-Profiling is providing a full ^{1}H-NMR-spectrum for each sample. In principle, all NMR spectrometers are suitable for this analysis. A 400 MHz spectrometer with proton optimized

detection and automatic sample changing was defined as the basis of the procedure. Strict SOP`s have been established for sample preparation, storage, measurement and processing.
For sample preparation only a predefined buffer (1M KH_2PO_4) has to be added. For cloudy sample a centrifugation has to be done prior buffer addition. Concentrates are diluted to reach a predefined Brix value. 540µl of sample material is mixed with 60 µl buffer (containing phosphate buffer in D_2O) to obtain a final sample pH of 3 (e.g. for fruit juice and wine).
The ^{1}H-NMR spectral acquisitions are carried out by using a modified version of the NOESYGPPRID pulse sequence, with standardized continuous wave pre-saturation of the water resonance (25Hz field), during the relaxation delay.
For fruit juice time domain data were collected into 64K data points. A recycle time of ~8 sec was covering a sweep width (SW) =20 ppm. An exponential window function, with line broadening (LB) = 0.3 Hz, was used prior to Fourier transformation of the data with zero filling into 64K frequency domain points.
Data processing with standard Bruker spectrometer software TopSpin was performed.
Baseline and phase correction were carried out using an automated processing program. Measurement procedure runs under complete control of a laboratory management system using barcodes for every sample.
For wine, acquisition conditions only vary in the solvent suppression mode, using a shaped pulse with 8 suppression frequencies on each individual solvent line with a presaturation field of 3 Hz per frequency.
Per registered spectrum, different interpretation routines are performed. Each interpretation of data is carried out by uni- and multivariate statistical analysis to reference models based on authentic samples (untargeted mode). The system is calibrated with a reference database containing spectra of representative samples covering the natural variability of the examined sample type. The quality of interpretation depends on representativeness and the number of reference spectra. The databases for the food analysis are built up using certified authentic samples of juices from the industrial process. All spectra have to be acquired under identical conditions to ensure comparability. For each sample a fully signed documentation including origin and condition of processed fruits, as well as the applied technology is available to guarantee traceability.
From the large range of available multivariate statistical methods applied in context of metabonomics data analysis (Vandeginste et al., 1998; Hastie et al., 2001), different possibilities of comparison can be used. A multitude of NMR spectral parameters can be entered into mathematical models. The combination of methods can improve sample characterisation and in some cases final judgement. Individual components are identified and quantified using information from a reference compound NMR spectral base (targeted mode).
Besides in-house developed scripts for MatLab, data analysis was performed mainly with the Bruker software package AMIX. For chemo metrical data analysis, spectra are segmented into equidistant chemical shift regions, the so-called buckets. Respective segment integrals are calculated in order to set up so called bucket tables for input into Principal Component Analysis, combined with discriminant analysis. Bucketing parameters were adjusted to the specific aspects investigated.

RESULTS AND DISCUSSION

The SGF Profiling for fruit juice represents a heterogeneous collection of cascading statistical models which can be applied consecutively to one single spectrum , see figure 1, such as specific models for multi fruit type separation, fruit type differentiation between citrus varieties (e.g. citrus sinensis and citrus reticulata), differentiation of product categories (e.g. orange juice and orange juice made from concentrate), or characterisation of compositional differences for two groups of similar products (e.g. apple juice concentrates from Poland and

China). Different answers presented as graphical or numerical results can be compiled in an analytical report by suitable software.
Only an exemplary extract of the possibilities can be presented here. In a cloud representation of PCA output as well as in classification, the position of the examined sample spectrum is indicated by a star.

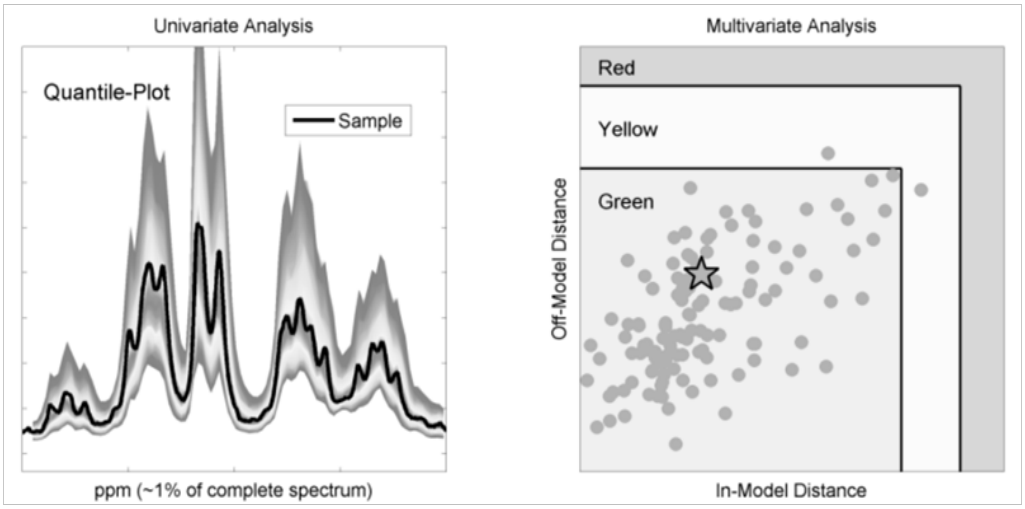

Figure 1. Univariate Analysis vs. Multivariate Analysis in NMR based Food Screening shown on orange juice sample. The univariate model is represented by the colour-coded quantile plot. The yellow and the red limits on the multivariate analysiswere calculated by Monte-Carlo/Cross-Validation analysis and represent the 95% and 99% confidence limit respectively.

An orange juice concentrate is from a Brazilian producer has been allocated to the common group, Orange/Mandarin and has been separated from other fruit types.

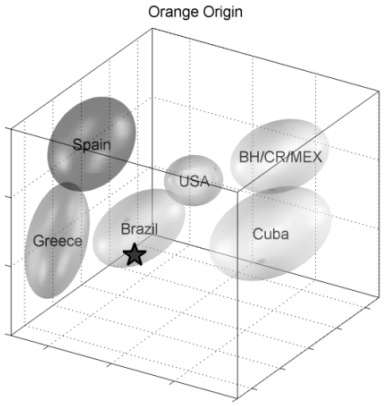

Figure 2. Orange Juice Origin as determined by rapid NMR screening

The same sample is projected to differentiation of geographic origin, which is a relevant commercial aspect with regard to price and quality. The control of product type is important in some cases, to detect economic frauds, e.g. if a label says direct juice, however in reality rediluted concentrate was used. Therefore a special model was developed to solve such question.
All models comparing different groups apply similarity calculations in order to classify any sample. Such models are mainly used to check declared specificities of any product in comparison with defined alternative possibilities. The Soft Independent Modelling of Class Analogy is comparing a spectrum only with one specific reference spectra set, in order to determine if spectral sequences with low variability in the reference group. This can indicate a deviation from the target population, without comparing to any defined alternative. The same spectrum, as per previous figures, is calculated here.

An addition of citrus reticulata (mandarin, clementine) in orange juice is not allowed in Europe. Such an addition is considered as an adulteration. A Ridge Regression curve is calculated based on pure orange and mandarin juice samples, for which spectra have been combined mathematically with different proportions. Value 0 corresponds to 100% pure citrus sinensis, value 1 to 100% pure citrus reticulata. In the laboratory, different mixtures of juices used for modelling, were prepared and measured with the calibration system. The share of reticulata is given on the x-coordinate. The results close to the linearity curve indicate good prediction and are in line with verification by DNA-testing and conventional analysis.
Similar models, as shown for orange juice products are developed for other juice products as well as for wine, honey and edible oil, too.
Furthermore, using the same spectra again, quantification can be carried out. Considering that analytical errors for both methods could be added when comparing NMR results and conventional analysis by enzyme test kit and/or HPLC, the NMR precision is more than sufficient for a pre-screening prior to conventional analysis.
Quantification of multiple compounds can be performed with one calibration and reference database to finished goods (bottled consumer goods). Furthermore, other models are transferable like the control of fruit content in products, with less than 100% fruit content such as drinks and nectars.
For better performance of compound identification, in case of signal overlap, a rapid 2D J-Resolved spectrum (Braun et al., 1998) is recorded as part of the standard routine.

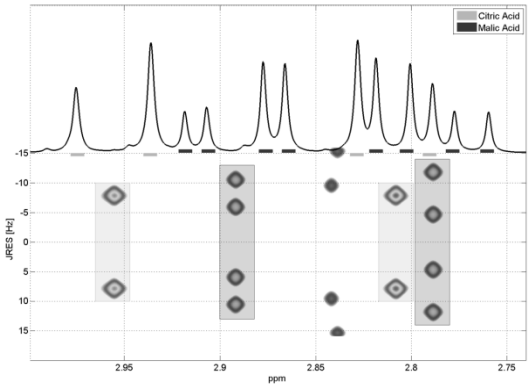

Figure 3. Rapid 2D J-Resolved spectrum recorded during the automatic measurement procedure. Upper part shows sub-region of 1D spectrum of pear juice sample mainly displaying overlap of peaks from malic and citric acid. Lower part shows subregion of JRES where coupling information and multiplicity information of malic acid peaks and citric acid peaks can be obtained from F1-dimension.

Figure 3 shows a region of the ID (NOESY) and 2D (JRES) spectra of a pear juice. Signals of Citric Acid (light grey) and Malic Acid (dark grey) are separated in the 2D-spectrum. With the information of JRES correct peak assignment it is possible in the 1D spectrum, however quantification from 1D is assisted by JRES.

Extension to food and drink materials other than fruit juice

Having established the statistical and quantification methodology, it is possible to expose the technology described for fruit juices to other food material by exchanging the underlying spectral database from juice for example to wine. The knowledge base for quantification in this case has to be modified as well to represent chemical shift variation of the compounds to be quantified, as they are observed in a different matrix, which can also produce different signal overlay problems. Applying the statistical technology to a set of about 800 German wines allows e.g. to differentiate variety, geographical origin and vintage year.
Figure 4 shows the analysis of the vintage year on a set of Riesling Wines from Rhineland-Palatinate in Germany. On the left side a 2-dimensional projection of the 4-dimensional space is shown for the years 2004 to 2008. On the right side the respective confusion matrix is shown, indicating the very low rate of false assignment even keeping in mind the still small sample cohort used (184 wines).

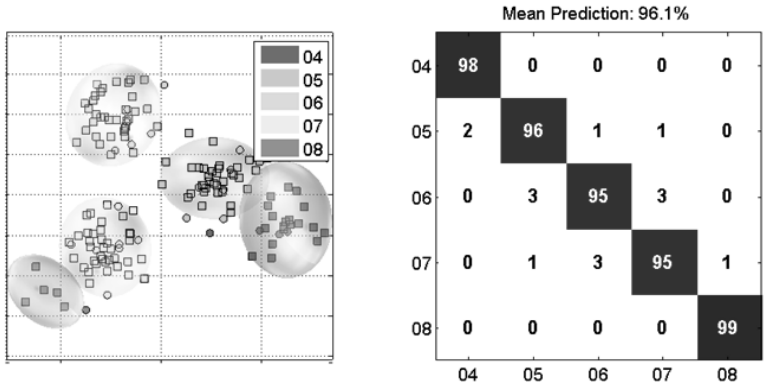

Figure 4: Statistical Analysis of the vintage year on 184 Riesling wines from Rhineland-Palatinate (Germany)

Melamine addition to baby milk powder was and still is a threat to the health of babies especially in China.
Added to pretend higher protein content, melamine was not detected for long time due to the targeted nature of food control methods used. Applying the NMR approach readily leads to the detection of melamine comparing to authentic baby milk powders from China (Lachenmeier et al., 2009). Figure 5 shows a quantile plot of the authentic model of milk powders dissolved in DMSO. The quantile plot can be interpreted as a superposition of all authentic sample spectra, the red colour indicates the average intensity at each point in the spectrum, while the dark blue colour indicates rare intensities in the dataset at the upper and lower limit. It can be seen, that the melamine signals clearly falls outside the range described by the normal model. When the same sample set was dissolved in H2O it could be shown, that sucrose was added to compensate the deviating sugar/nitrogen ratio.

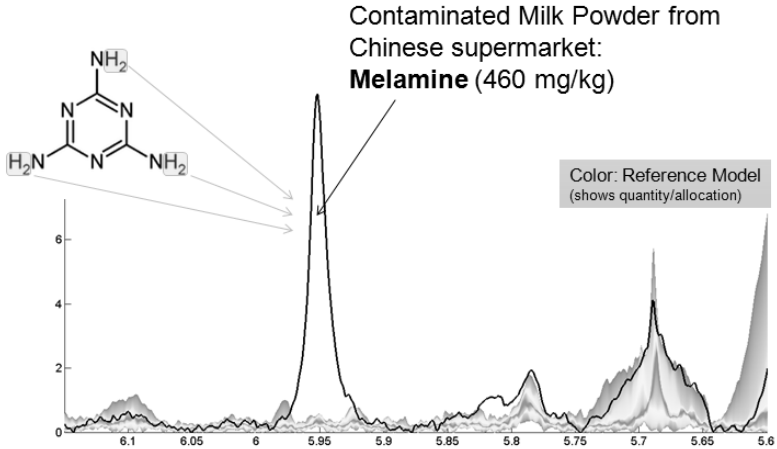

Figure 5: Comparison of contaminated milk powder NMR spectrum in DMSO to quantile plot of an authentic sample set

The two examples given indicate the potential of NMR in food quality and safety, being transferable from one material to another applying the corresponding normal sets. Investigations have also been made on olive and other edible oils as well as honey and mozzarella cheese.

CONCLUSIONS

SGF-Profiling is a NMR-based high throughput screening for fruit juices. The combination of chemo metric modelling with molecule specific signal treatment makes it a powerful tool for fruit juice quality assessment. Specific quality aspects and adulterations can be identified by applying a number of statistical tests on the same spectrum. Fruit and product type differentiation, adulteration by sugar or acid addition, geographical origin and fruit mixtures are predictable. Moreover, a list of substances being present in fruit juice is quantifiable. The application of SGF-Profiling in routine controls is enhancing the control density and the amount of detected quality deviations. The pre-selection carried out allows choosing well targeted conventional analysis for a sample. In many cases, it can save higher analytical costs. Therefore the SGF-Profiling is useful and extremely economic in cases where samples need a very fast analytical answer and where a large number of samples should be tested and can be transferred to other food materials as shown.

ACKNOWLEDGEMENTS

The Riesling Sample Set was obtained from Prof.Fischer from DLR Neustadt Germany

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THE FATTY ACIDS AND ACYLGLYCEROLS CONTENT AND COMPOSITION OF CHICKPEA FLOUR

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ABSTRACT: In this paper the composition and content of fatty acids and acylglycerols of lipids from chickpea flour were examined. The chickpea flour was obtained by milling seeds to an average particle size of 0.3 mm. The lipids are obtained by trichlorethylene duplicate extraction and by using reflux at solvent boiling temperature. For the content and composition of chickpea flour fatty acids and acylglycerols analysis, the GC and HPLC method, respectively, were performed. In order to obtained the fatty acids methyl esters, the lipids were alkaline hydrolyzed and methylated. The content of lipid was 3.11 g per 100 g of chickpea flour and fatty acids composition was palmitic 12.18%, stearic 2.47%, arachidonic 1.10%, behenic 0.52%, oleic 37.77% and linoleic 42.14%. The result showed the chickpea flour contained 16.27% of total saturated fatty acids, 38.28% of total monounsaturated fatty acids and 42.14 % g of polyunsaturated fatty acids, so the total unsaturated fatty acids was 80.14%. The ratio of total unsaturated to saturated fatty acids content was 4.93, and as this ratio is higher than ratio previously obtained for wheat flour, it can be concluded that chickpea flour addition to the wheat flour in dough, as well as in final product, will change this ratio in benefit to unsaturated fatty acids. By HPLC analysis it was obtained that the lipids had the highest content of triacylglycerols (57.48%), while the content of diacylglycerols was 36.66% and monoacylglycerols, 5.51%.
Key words: *fatty acids, acylglycerols, chickpea flour*

INTRODUCTION

The chickpea (*Cicer arietinum* L.) known also as ceci bean, garbanzo bean, chana, sanagalu Indian pea and Bengal gram, is alegume plant of the family *Fabaceae.* It is one of the earliest cultivated vegetables and there are the remains found in the Middle East, 7,500 year old. The chickpea seed is an excellent source of protein ranging between 12 and 30% and healthy carbohydrate known as dietary fiber (Sotelo et. al., 1987; Saleh and Tarek, 2006). Due to these components it is a great food especially for diabetics and insulin-resistant individuals. Chickpea also can help in lowering cholesterol and improving the blood sugar levels (Marray et al., 2005). Much work has been done to improve protein quality and amino acid composition in several crops (Mosse and Baudet, 1983; Monti and Grillo, 1983). The chickpeas provide an excellent source of folic acid and minerals such as iron, copper, zinc and magnesium, as well as phenolic compounds (Segev, et al., 2010).
Oil content, including essential fatty acids is the third important organic component of chickpea. According to genotype and environmental factors, oil content had wide range between 3.8 and 10% (Nikolopoulou et al., 2006). Linolenic, oleic and palmitic acids are the major fatty acids of chickpea oil (Ling and Robinson 1976). The previous research indicated that cultivation method, growing conditions, environmental factors and planting time (Nikolopoulou et al., 2006; Gül et al. 2011), affect on yield and fatty acid composition in chickpea seeds. Recently there are increasing interests for fatty acids and acylglycerols composition since there are reports about fatty acid influence, especially of n-3 and n-6 fatty acids and their ratio, on human health (Simopolous, 2002; Simopolous, 2008). The influence

includes benefits related to cancer, inflammatory bowel disease, rheumatoid arthritis, and psoriasis (Rose et al., 1999; Connor, 2000).
In general, the lipids content in seed of leguminous plant is considerably and the lipids have an important physiological role in food quality, such as dough rheology (Dobraszczyk and Morgenstern, 2003). The addition of flour of leguminous plant seeds (Nikolić and Lazić, 2011,) or other plant to wheat flour, changes the dough rheology and lipid composition (Nikolic, et al., 2011). In order to investigate the effect of chickpea flour addition to the wheat flour on dough rheology and its lipid composition, these results are needed. They represent the first step to thataim and are important for predicting the potential benefits for nutrition value of final product.
The aim of the study presented in this paper was to investigate fatty acid composition and acylglycerols of lipids from chickpea flour with the emphasis on total unsaturated and saturated fatty acids content and their ratio.

MATERIAL AND METHODS

Plant material

The decorticated and fried chickpea seeds, originated from Iran, grown in 2009 year, and were bought from the local market in Leskovac, Serbia. The chickpea flour was obtained by milling of chickpea seeds and sieving through a 0.25 mm riddle.

Lipids content determination and isolation

The lipids content was determined by trichlorethylene duplicate extraction, for the same sample, by using reflux (1:20 w/v at boiling temperature, 60 minutes). The extracts were combined and 3 ml were dried at 110 ^{o}C to a constant weight and the dry residue content was read out on the analyzer display (Scaltec SMO 01, Scaltec instruments, Germany). For lipids isolation, the rest of combined n-hexane extracts was evaporated under vacuum.

Fatty acid composition determination by GC analysis

For GC analysis, fatty acids methyl esters were prepared. The lipids were alkaline hydrolyzed and methylated by methanol and BF_3 as catalysts. The final fatty acids methyl esters concentration was about 8 mg/ml in heptane. For obtaining a methyl esters GC spectra, the HP 5890 SERIES II GAS-CHROMATOGRAPH, HP with FID detector and 3396 A HP integrator was used. Column was ULTRA 2 (25m x 0.32mm x 0.52 μm) (Agilent Technologies, Wilmington, USA), injector temperature of 320 ^{o}C, and injector volume of 0.4 μl. The carrier gas was He at a constant flow rate of 1 ml/min. The flame ionization detector was at 350 ^{o}C and split ratio was 1:20. Oven temperature was initially 120 ^{o}C and was maintained at 120 ^{o}C, for 1 min, then increased by 15 ^{o}C/min until 200 ^{o}C, increased by 3 ^{o}C/min until 240 ^{o}C, increased by 8 ^{o}C/min until 300 ^{o}C and maintained at 300 ^{o}C for 15min. The fatty acids were identified by comparison of retention times of the lipids components with those of standards.

Acylglycerols composition determination by HPLC analysis

For HPLC analysis, Holčapek et al. (1999) modified HPLC method and the Agilent 1100 High Performance Liquid Chromatograph, a Zorbax Eclipse XDB-C18 column: 4.4 m x 150 mm x 5 μm (Agilent Technologies, Wilmington, USA), and an UV/ViS detector were used. The samples of the reaction mixture were dissolved into a mixture of 2-propanol: *n*-hexane, 5:4 v/v and filtered through 0.45 μm Millipore filters. The flow rate of binary solvent mixture (methanol, solvent A, and 2-propanol/*n*-hexane, 5:4 by volume, solvent B) was 1 ml/min with a linear gradient (from 100% A to 40% A+ 60% B in 15 min). The column temperature was held constant at 40 ^{o}C. The components were detected at 205 nm. The monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG) were identified by comparing the retention times of the lipids components with those of standards.

Statistical analysis

STATISTICA, version 5.0 software was used to perform the statistical analysis: the means and standard deviations and cluster analysis. The means and standard deviations were obtained by Descriptive Statistics, marking the Median & Quartiles and Confirm Limits for Means. For cluster analysis the Euclidean method with the complete linkage was used.

RESULTS AND DISCUSSION

The lipids content in investigated flour from chickpea seeds was 3.11 g per 100 g of chickpea flour and it is lower than those found in literature data (Nikolopoulou et al., 2006). The results of lipid profile analysis, the free fatty acids, total saturated fatty acids (TS), total monounsaturated fatty acids (TMU), total polyunsaturated fatty acids (TPUS), total unsaturated fatty acids (TUS), monoacylglycerols (MAG), diacilglycerols (DA) and triacylglycerols (TAG) are presented in Table 1. The HPLC chromatogram of acylglycerols is presented in figure 2. The content of acylglycerols was determined by measuring the peak area from 3.445 to 4.580 min, for MAG, peaks area from 5.276 to 8.677 min, for DAG and peaks area from 10.907 to 15.815 min, for TAG.

Table 1. Fatty acid and acylglycerol composition (% w/w) of chickpea seed flour

Fatty acid composition obtained by GC analysis	
Fatty acid	**Content (%)**
Palmitic acid (16:0)	12.18± 0.1
Stearic acid (18:0)	2.47± 0.09
Arachidic acid (20:0)	1.10± 0.09
Behenic acid (22:0)	0.52± 0.06
Palmitoleic acid 16:1)	0.51± 0.06
Oleic acid (18:1)	37.77±1.32
Linoleinic acid (18:2)	42.14±2.32
TS (%)	16.27±0.2
TMUS (%)	38.28±1.56
TPUS (%)	42.14±1.75
TUS (%)	80.14±2.37
Ratio TUS toTS	4.93
Acylglycerol composition obtained by HPLC analysis	
Acylglycerol	**Content (%)**
MAG	5.51±0.09
DAG	36.66±1.69
TAG	57.84±2.78

Results are given as mean ± standard deviation (n = 3);
TS –total saturated fatty acids;TMU – total monounsaturated fatty acids; TPUS –total polyunsaturated fatty acids;TUS – total unsaturated fatty acids; MAG-monoacylglycerols, DAG-diacilglycerols; TAG-triacylglycerols

Fatty acids composition was: palmitic 12.18%, stearic 2.47%, arachidonic 1.10%, behenic 0.52, oleic 37.77% and linoleic 42.14%. The result showed the chickpea flour contained 16.27% of total saturated fatty acids, 38.28% of total monounsaturated fatty acids, 42.14% of polyunsaturated fatty acids, so the total unsaturated fatty acids was 80.14%, and ratio of total unsaturated to saturated fatty acids was 4.93. The previously obtained data of GC analysis (Nikolić, et al., 2011) showed this ratio was 3.71. Based on these data, it can be concluded that chickpea flour addition to the wheat flour in dough will change unsaturated to saturated fatty acids ratio in benefit to unsaturated fatty acids.

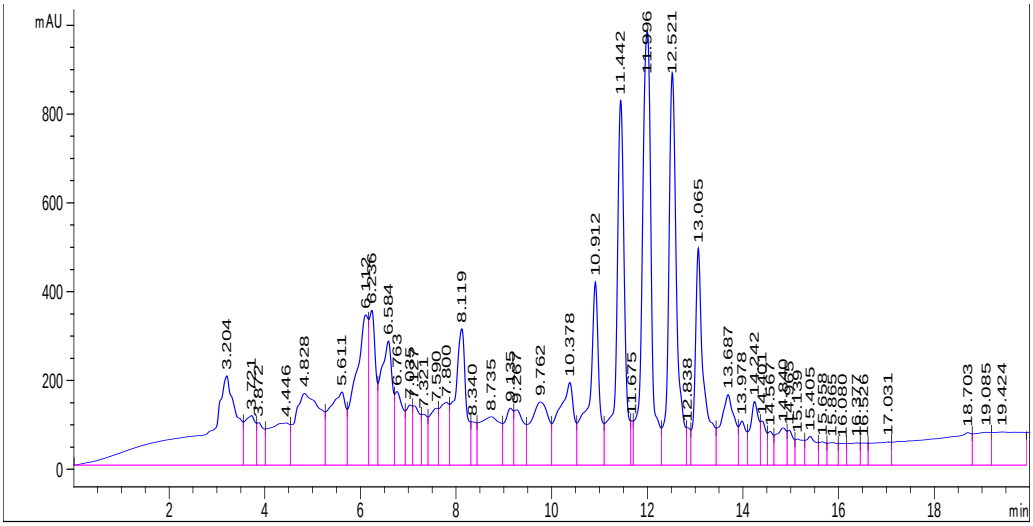

Figure 1. The HPLC chromatogram of acylglycerols of lipids from flour of chickpea seeds

By HPLC analysis, in lipids from chickpea flour, the highest content had triacylglycerols (57.48%), while the content of diacylglycerols was 36.66% and monoacylglycerols, 5.51%.
The diagram of Euclidean distances based on eight variables (palmitic-PAL, oleic -OLE and linolenic -LIN fatty acid content, total saturated-TS and total unsaturated fatty acids -TUS content and content of mono- (MAG), di- (DAG) and triacylglycerols (TAG)) are shown in figure 2. Results of cluster analysis indicate, in total saturated fatty acids the palmitic acid had the main part, the oleic acid in diacylglycerols, while in total unsaturated fatty acids it was the linolenic acid.

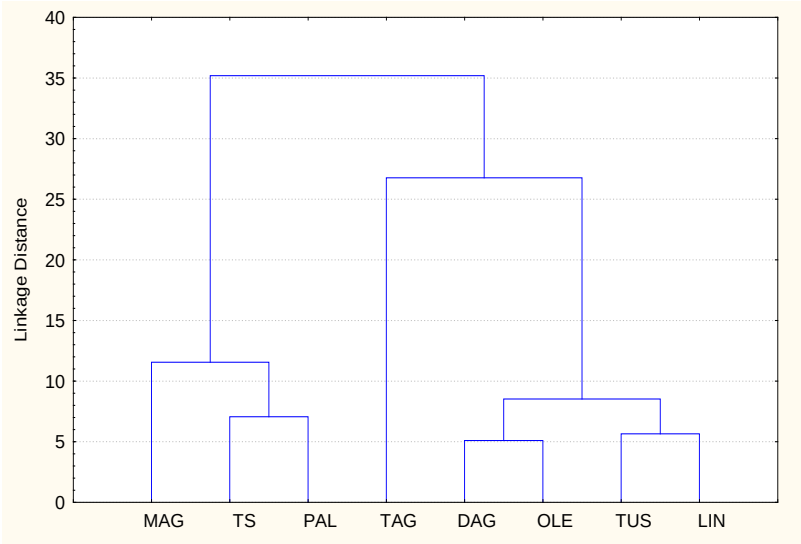

Figure 2. The Euclidean distances for components of lipids from flour of chickpea seeds

In investigated sample of flour from chickpea seeds, the content of lipid was 3.11%. The fatty acids profile composed from palmitic (12.18%), stearic (2.47%), arachidonic (1.10%), behenic (0.52%), oleic (37.77%) and linoleic (42.14%) acid. The ratio of total unsaturated to total saturated fatty acids content was 4.93. As this ratio is higher than ratio previously obtained for wheat flour, it can be concluded that chickpea flour addition to the wheat flour will change unsaturated to saturated fatty acids ratio in dough, in benefit to unsaturated fatty acids. The analysis of acylglycerols content showed the highest content had triacylglycerols (57.48%), while the content of diacylglycerols was 36.66% and monoacylglycerols, 5.51%.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project Grant No.172047 „Natural products from plants and lichens: isolation, identification, biological activity- the possibility for final application" by the Ministry of Education and Sciences Republic of Serbia.

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SUITABILITY OF THIOBARBITURIC ACID METHOD FOR ASSESSING LIPID OXIDATION IN PORK, OSMOTICALLY DEHYDRATED IN SUGAR BEET MOLASSES

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ABSTRACT: Thiobarbituric acid (TBA) reaction for measuring secondary lipid oxidation products, was evaluated for interferences by ingredients of sugar beet molasses, used for osmotic dehydration. Pork meat was dehydrated in molasses solution and stored for 105 days at 4^{o}C, under the modified atmosphere conditions, or under the atmospheric conditions at 4^{o}C and 20^{o}C for 30 days. The effectiveness of different TBA methods was measured by aqueous acid extraction method (EM) under different conditions of incubation: 1) boiling or 2) room temperature, with 20 mM TBA, and 3) at 40^{o}C, with 80 mM TBA. The distillation TBA method (DM) was employed to evaluate the interferences found in EM, and low TBA values were obtained in all TBA method procedures. Primary lipid oxidation products were, also, low, except for microbiologically contaminated samples with increased water activity value, where elevated peroxide values, but low TBA values were obtained. The data are suggestive of oxidative stability of osmotically dehydrated pork meat under storage in modified atmosphere and refrigerated conditions. TBA EM may be the inadequate method for the analysis of oxidative deterioration of meat samples dehydrated in sugar beet molasses, due to interferences which cause erroneously high value of TBA, detected as absorption at: max. 350 nm and 450-460 nm, present in dehydrated meat and sugar beet molasses itself, which are overlapping the pink peak (max. 532 nm), characteristic for malondialdehyde. TBA EM procedure at low incubation temperature (40^{o}C), and elevated TBA concentration (80 mM), was the most sensitive of analyzed TBA methods.
Key words: *TBA test, pork, osmotic dehydration, molasses*

INTRODUCTION

Osmotic dehydration (OD) is one of the most relevant methods of food dehydration based on partial removal of water from the food material by direct contact of product with hypertonic solutions of salts, sugar, acids, etc., (Raoult-Wack, 1994). Reduction in moisture and water activity of the food matrix lead to the reduction in microbial growth (Rastogi et al., 2002) and differences in lipid oxidation status (Singhal et *al.*, 1997). Lipid oxidation involves the degradation of polyunsaturated fatty acids (PUFA) and the production of primary (hydroperoxides) and secondary lipid decomposition products (Claxson et al., 1994). Free fatty acids (FFA), peroxyde values (PV) and thiobarbituric acid-reactive substances (TBA-RS) have been most commonly used determinants of the degree of lipid oxidation. Brining and drying of meat generally increase FFA, PV and TBA-RS (Khuntia et al., 1994). The assessment of primary lipid oxidation products, using PV, is limited because peroxides are unstable intermediate products in the formation of carbonyl compounds. The thiobarbituric acid (TBA) test is widely used to evaluate secondary lipid oxidation products in meat. Malondialdehyde (MDA), a secondary oxidation product of PUFA with three or more double bonds, reacts with TBA to form a stable pink chromophore with maximal absorbance at 532 nm (TBA-RS_{532}). Aqueus acid extraction method (EM) and the distilation method (DM) are the most frequently applied TBA tests in meat research. The EM may be considered as more suitable method for estimating the MDA, because the meat itself is not exposed to heat treatment. Certain compounds interfere with the reaction between TBA and MDA: sugars, water-soluble proteins and peptides, DNA, volatile aldehydes different to MDA, pigments,

amino acids, nitrites, metals and compounds with similar spectral properties to that of the TBA–MDA adduct. Numerous method modifications were developed to reduce these interfering reactions (Ganhao et al., 2007) (Fernandez et al., 1997) (Wang et al., 2002).
Recent research has shown that usage of sugar beet molasses as hypertonic solution improves osmotic dehydration processes (Koprivica et al., 2009). Molasses is an excellent medium for osmotic dehydration, due to the high dry matter (65-80%) and enrichment of the food material in minerals and antioxidants (Koprivica et al., 2009) (Guimaraes et al., 2007). High level of sucrose (44-54%), other sugars and their degradation products in molasses (El-Geweley, 1997), used in osmotic dehydration of meat, could generate interfering yellow chromagen (max. 450-460 nm) overlapping the pink peak (max. 530-537 nm) of TBA-MDA aduct. Yellow chromagen is formed by a variety of aldehydic compounds reacting with TBA (Fernandez et al., 1997). The reaction between TBA and MDA is more specific at room temperature than at boiling temperature. Hence, the interfering colorings can be reduced by incubating TBA and MDA at lower temperatures, while the reaction time can be reduced by increasing the concentration of TBA concentrations from 20 mM to 80 mM. The modified method can be used to measure MDA in the presence of high concentrations of interfering sucrose (higher than 4%), or other similar interfering agents (Wang et al., 2002).
The objective of this study was to choose the appropriate method for TBA-RS measurement in pork meat, osmotically dehydrated in sugar beet molasses which contained 4-13% of sucrose. The difficulty to determine the optimal conditions for the release of MDA from its bound forms in muscles without using strong acidic conditions and heating, which, could destabilize the MDA-TBA complex (Fernandez et al., 1997), was suggestive of combining mild extraction procedure (Wang et al., 2002) with different incubation temperatures and TBA concentrations in EM. The effectiveness of different EM-TBA was evaluated by applying the DM-TBA (Tarladgis et al.,1964).

MATERIALS AND METHODS

Osmotic dehydration

Fresh pork (Musculus brachii) of normal pH (6.05), was cut into approximately 1x1x1 cm ($1cm^3$) cubes and dehydrated in solutions of sugar beet molasses (81,95 ^{0}Brix) at 22°C for 5 hours.

Modified atmosphere packaging (MAP)

Osmotically dehydrated meat (100g/sample) were put in packaging molds under modified atmosphere (30% CO_2 : 70% N_2. Packaged meat samples were stored at 4 ± 0.5 ^{o}C for 105 days and at 4 ± 0.5 ^{o}C or 20±1 ^{o}C for 30 days.

Water content

Water content of the osmotic dehydrated samples was determined by the ISO 1442:1997.

Water activity (a_w)

a_w of the osmotic dehydrated samples was measured using a water activity measurement device (TESTO 650, Germany) with an accuracy of ±0.001 at 25ºC.

pH measurements

Was determined by ISO 11289:1993.

Acid value (free fatty acids, FFA)

Was measured after cold lipid extraction of samples (using chloroform), according to ISO standard method for determination of acid value and acidity in oils and fats of vegetable and animal origin, (ISO 660:1996). Acidity was expressed as acid value (mg KOH/g of sample) or as % FFA (% of oleic acid).

Peroxide value
Was measured after cold lipid extraction of samples (using chloroform), according to ISO standard method for determination of peroxide value in oils and fats of vegetable and animal origin (ISO 3960/1998). Peroxide value was expressed as mmol O_2/kg of sample.

TBA test
Secondary lipid oxidation products were determined using both aqueous acid extraction TBA method (EM), (Wang et al., 2002), and a distilation method (DM) Tarladgis et al. (1964). The incubation conditions in EM for TBA-reactive substances **(**TBA-RS) were: A) boiling temperature/25 min with 20 mM TBA, B) 40^{o}C/70 min, with 80 mM TBA and C) 20 ^{o}C/20 h with 20 mM TBA. D) The steam distillation TBA method (DM) was employed to evaluate the interferences with the spectrophotometric measurement at around 532 nm found in EM. TBA-RS values were calculated by multiplying the absorbance values at 532 nm by a constant coefficient K_{med}. K_{med} value was from standard curves and known dilutions of MDA standard (1, 1, 3, 3 tetrametoxypropane, TMP), and expressed as mg MDA/kg of sample.

Statistical analysis
MicroSoft Excel software (MicroSoft Office 2003) was used for statistical analyses. All measurements were performed in triplicate.

RESULTS AND DISCUSSION

Water content, water activity and pH in pork meat after osmotic dehydration
Dehydration in sugar beet molasses solution caused a significant moisture loss in meat samples, from initial water content of 75.957±0.045%, before osmotic dehydration, to 40.433±0.244%-47.13±0.030, after osmotic dehydration, during 105 days of observation.
The water activity (a_w) values decreased with the osmotic treatment from 0.938±0.002 to 0.872±0.00, which resulted in the microbiologically more stable product (Šuput, personal communication). After approximately 60 days, the a_w values raised to the final values of 0.894±0.001 (Fig.1). The enhancement of the a_w values coincided with the increase in total bacterial count after 60 days of observation (Šuput, personal communication).

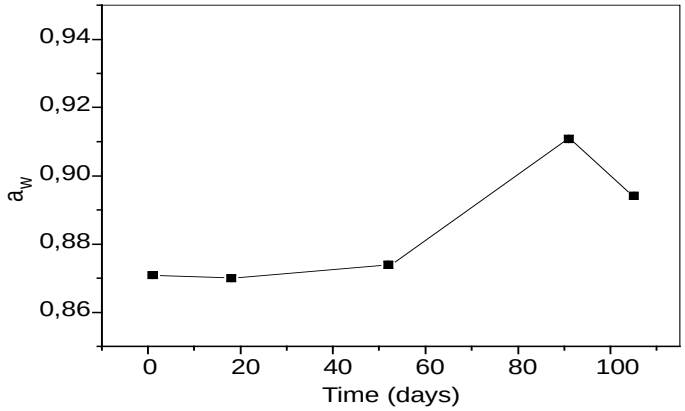

Figure 1. Water activity (a_w) kinetics during storage of pork meat, dehydrated in sugar beet molasses and stored in MAP at 4^{o}C.

pH values of osmotically dehydrated meat decreased during first 60 days of observation from 6.693±0.11 to 6.387±0.006. pH decreases in MAP more that in any other packaging, as common gases used in MAP are carbon dioxide (to inhibit bacterial growth), and nitrogen (to avoid oxidation of fats and pack collapse). Final increase of pH to 6.98±0.004 was probably caused by modified atmosphere changes.

Acid value (free fatty acids) and peroxide value

At refrigeration temperatures, free fatty acids are enzymatically released from neutral lipids and phospholipids. FFA in pork meat at 2°C are caused by lipases, produced by spoilage bacteria (Chung-Wang et al., 1997). In our study, the concentration of free fatty acids remained low, in the range of 17.64±0.5 to 22.3±0.54 mgKOH/g of sampe (0.049-0.63 % FFA). Primary lipid oxidation products were, also, low (peroxide value=0), except for microbiologically contaminated samples with increased water activity values, where elevated peroxide values (more than 12 mg O_2/kg were obtained).

TBA analysis

The effectiveness of different TBA tests in minimizing the interferences of sugar molasses, used as osmotic solution, was measured by aqueous acid extraction method (EM) under different conditions of incubation (A, B and C). Interferences, which cause erroneously high value of TBA-RS in meat samples dehydrated in molasses, were detected as absorption at 350 nm, 450-460 nm, and 532 nm. These spectrophotometric interferences, found also in diluted molasses solution, are overlapping the pink peak (532 nm), characteristic for MDA, (Fig. 2 and Fig. 3). The reaction of molasses with TBA was dependent on concentration and maximal at 100°C, (Fig. 2, scan 2 and 4), and than slowed when the incubation temperature was decreased, (Fig. 3, scan 2 and 4). It is known that sucrose reacts with TBA, generating a yellow adduct with maximum absorbance at 450 nm, when the incubation temperature is above 50 °C (Fig. 3, scan 5) and interfering reaction stopped when the incubation temperature was decreased to 40°C (Fig. 3, scan 5) (Wang et al., 2002). Since interferences in 20% molasses solution (with approximately 50% sucrose) was diminished, but not completely reduced, as in 10% sucrose, when the incubation temperature was decreased to 40°C (Fig. 3, scan 4 vs. scan 5), it is evident that some other TBA-reactive compounds, possibly antioxidants, (Guimaraes et al., 2007) may be present in the sugar molasses, which can lead to interferences with the spectrophotometric measurement and hence, cause an overestimation of the results. For such problematic samples, the DM TBA method may be a reliable option to assess TBA-RS values. The oscillations in TBA-RS values, obtained in DM method, (Fig. 4. D), are, however, suggestive of interferences from molasses. Molasses contain more than 40 volatile products, mainly aldehydes (El-Geweley, 1997), which could be released in DM procedure.

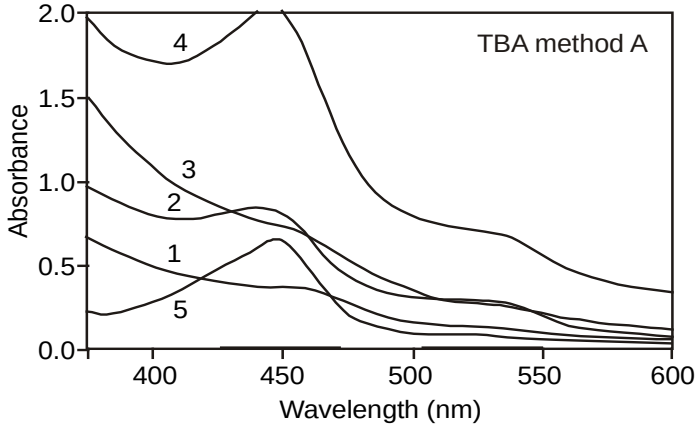

Figure 2. Scans of sugar beet molasses and sucrose absorbance in TBA method A (EM, 100°C/25 min, 20 mM TBA). Scans: 1) 10% molasses, before TBA reaction; 2) 10% molasses, after TBA reaction; 3) 20% molasses, before TBA reaction; 4) 20% molasses, after TBA reaction, 5) 10% sucrose after TBA reaction.

TBA-RS values were low in all analyzed methods, which are indicative of oxidative stability of osmotically dehydrated pork meat under storage in modified atmosphere and refrigerated conditions. Under storage at atmospheric conditions, in TBA method B, the most significant increase in TBA-RS values was found in meat samples stored 30 days at 20°C, (Fig. 5). It must be, however, taken into consideration that in dehydrated meat, mayor part of MDA may

be in volatilized form, which can cause difficulties in MDA detection (Fernandez et al., 1997). TBA values of microbiologically contaminated samples with increased water activity and peroxide values, were low in DM TBA method, in accordance with possible lipolytic effect of bacteria (Fig. 5, D) (Chung-Wang et al., 1997).

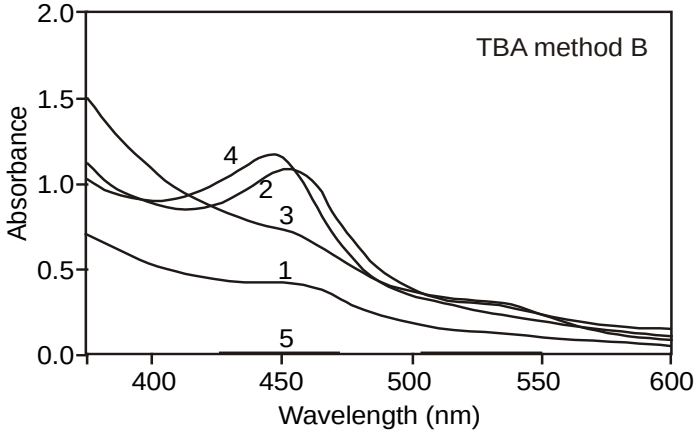

Figure 3. Scans of sugar beet molasses and sucrose absorbance in TBA method B (EM, 40°C/70 min, 80 mM TBA). Scans: 1) 10% molasses, before TBA reaction; 2) 10% molasses, after TBA reaction; 3) 20% molasses, before TBA reaction; 4) 20% molasses, after TBA reaction, 5) 10% sucrose after TBA reaction

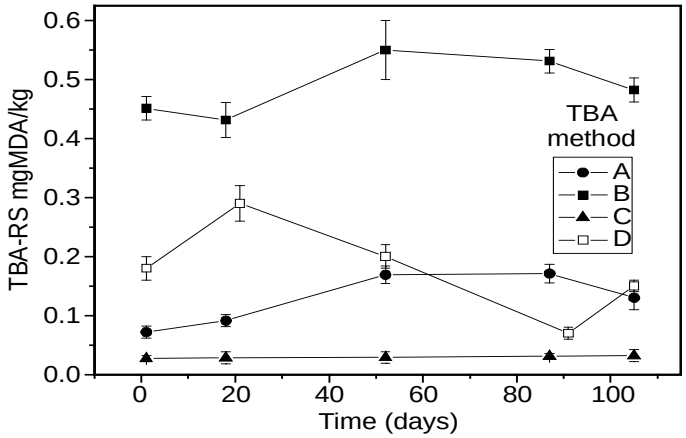

Figure 4. Thiobarbituric acid reactive substances (TBA-RS) kinetics during storage of pork meat, dehydrated in sugar beet molasses and stored in MAP at 4°C. TBA method*:

A) EM,100°C/25 min, 20 mM TBA
B) EM, 40°C/70 min, 80 mM TBA
C) EM, 20°C/20 h, 20 mM TBA.
D) DM, steam distillation

**Absobance values at 532 nm before TBA reaction was substracted.*

CONCLUSION

Pork meat was osmotically dehydrated in sugar beet molasses and stored for 105 days, at 4°C, under the modified atmosphere (MAP), or under atmospheric conditions at 4°C or 20°C for 30 days. The interferences of ingredients of sugar beet molasses, presumably sugars and sugar degradation products, are evaluated in TBA reaction for measuring secondary lipid oxidation products. The effectiveness of three TBA tests in minimizing the interferences of molasses ingredients was measured by aqueous acid extraction method (EM) under different conditions of incubation. The distillation TBA method (DM) was employed to evaluate the interferences found in EM, and low TBA values were obtained in all TBA procedures. Primary lipid oxidation products were, also, low, except in microbiologically contaminated samples with increased water activity values, where elevated peroxide values, but low TBA values

were obtained. TBA-EM may be the inadequate method for the analysis of oxidative deterioration of meat samples dehydrated in molasses, due to interferences which cause erroneously high values of TBA, detected as absorption at: 350 nm and 450-460 nm, present in dehydrated meat and sugar beet molasses itself, which are overlapping the pink peak (max. 532 nm), characteristic for MDA. TBA-EM procedure at low incubation temperature (40^oC) and elevated TBA concentration (80 mM) was the most sensitive of analyzed TBA methods. Low PV and TBA values in all analyzed methods are indicative of low lipid oxidation in osmotically dehydrated pork meat under storage in modified atmosphere at 4^oC.

ACKNOWLEDGEMENTS

This work is part of project no. TR-31055, suported by the Ministry of Education and Science Republic of Serbia.

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CADMIUM CONTENTS IN THE LIVER AND KIDNEY FOR FIVE PUREBRED PIGS FROM VOJVODINA

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ABSTRACT: The content of cadmium (Cd) was investigated in liver and kidney for five purebred pigs (Large White – LW, n = 48; Landrace – L, n = 48; Duroc – D, n = 48; Hampshire – H, n = 48 and Pietrain – P, n = 48), produced in Vojvodina. This element was determined by flame atomic absorption spectrometry after mineralization by dry ashing. The difference in the Cd content among the five purebred pigs was not significant in the analysed liver (F = 1.191; P = 0.315) and kidney (F = 0.867; P = 0.484) tissues. Pigs kidney Cd content was significantly higher (F = 461.650; P = 0.000) than liver. The order of the purebred pigs regarding Cd content in the liver samples in mg/kg was: H (0.057–0.261, on average 0.146) > LW (0.052–0.268, on average 0.138) > D (0.031–0.252, on average 0.133) > L and P (0.029–0.270 and 0.039–0.227, on average 0.124). The average Cd content in all investigated liver samples was 0.133 mg/kg. The order of the purebred pigs regarding Cd content in the kidney samples in mg/kg was: L (0.173–1.160, on average 0.412) > D (0.178–1.060, on average 0.398) > P (0.180–1.012, on average 0.367) > H (0.203–0.649, on average 0.366) > LW (0.168–0.654, on average 0.361). The average Cd content in all investigated kidney samples was 0.381 mg/kg. The maximum Cd content found in the liver samples was significantly below maximum level (0.5 mg/kg), while only 2.1% (n = 5) of kidney samples slightly exceeded the maximum level (1.0 mg/kg) set by EU and Serbian legislation. Obtained Cd contents in analysed liver and kidney tissues indicate Cd availability in the local agricultural environment in Vojvodina.
Key words: *pigs, liver, kidney, cadmium*

INTRODUCTION

Cadmium is a heavy metal found as an environmental contaminant, both through natural occurrence and from industrial and agriculture sources. The metal has been reviewed by the International Register of Potentially Toxic Chemicals of the United Nations Environment Programme. As a result, it has been included on the list of chemical substances and processes considered to be potentially dangerous at the global level (IRPTC, 1987). It is toxic, teratogenic, mutagenic and carcinogenic to most organisms (Robards and Worsfold, 1991).

Food is the most important source of cadmium exposure in the general non-smoking population in most countries (Andrée et al., 2010; Järup and Åkesson, 2009; Järup, 2003; Robards and Worsfold, 1991; Doyle and Spaulding, 1978). Cadmium is likely to accumulate in the food chain (Dave Oomah et al., 2007; Oskarsson et al., 2004; Lindén et al, 2003; Sharma et al., 1982). In mammals, cadmium is virtually absent at birth but accumulates with time, especially in the liver and kidney (Andrée et al., 2010; Järup and Åkesson, 2009; Sapunar-Postružnik et al., 2001; Lindén et al., 1999; Robards and Worsfold, 1991; Henke et al., 1970). Cadmium is efficiently retained in liver and kidney with a very long biological half life ranging from 10 to 30 years (Andrée et al., 2010; Järup and Åkesson, 2009; Friberg et al., 1974).

The World Health Organisation (1992) has recommended that the provisional tolerable weekly intake (PTWI) of cadmium not exceed 0.4–0.5 mg per person or 0.007 mg/kg body weight.

The cadmium maximum levels in food have been regulated in Republic of Serbia by national legislation (Serbian Regulation, 1992a, 1992b, 2002), which is in accordance with legislation of European Union (Commission Regulation (EC) No 1881/2006). The maximum levels are 0.05, 0.5 and 1.0 mg/kg (wet weight) for pig meat, liver and kidney, respectively.
Pig meat is the most widely consumed meat in the EU (Williamson et al., 2005), as well as in Serbia, and the consumption has been steadily increasing. The Autonomous Province of Vojvodina (the northern part of the Republic of Serbia) is a region where the number of animals of the porcine species and the production of pork meat are of high economic importance. Over 30 percent of the total number of pigs slaughtered annually in Serbia comes from Vojvodina. Five purebred pigs (Large White, Landrace, Duroc, Hampshire and Pietrain) and their crosess are used for commercial pork production. In (cross)breeding programme Large White and Landrace are used as female lines and Duroc, Hampshire and Pietrain are used as male lines.
The aims of this study were: (i) to obtained the cadmium levels of liver and kidney from pigs in Vojvodina; (ii) to investigate the potential differences in cadmium levels of liver and kidney among five purebred pigs, used nowadays in Vojvodina for pork production; (iii) to compare the obtained values with the maximum levels set by European Commission, i.e. by national legislation. The overall objective is to produce high quality pork to ensure the competitiveness of Vojvodian pork in the international meat markets.

MATERIALS AND METHODS

Animals, sampling and preparing

In this study five purebred pigs (castrates males and females) were used: Large White (LW), n = 48; Landrace (L), n = 48; Duroc (D), n = 48; Hampshire (H), n = 48; and Pietrain (P), n = 48.
The pigs were fattened at the production farms in the northern part of the Republic of Serbia (Autonomous Province of Vojvodina). The pig fattening involved the following phases: starting period (from 15 to 25 kg), growing period (from 25 to 60 kg) and finishing period (from 60 to 110 kg). The diets were based on locally produced corn and soybean meals, and were formulated to meet the nutrient requirements (National Research Council, 1998) for the different growth phases. The finishers were housed in pens with fully slatted floor and 0.80 m^2 space allocation per pig. Each pen contained 10 animals. The environmental temperature in the building was 22°C. All pigs had ad libitum access to a diet and water.
Animals were randomly selected during one year period. The pigs were slaughtered at a live weight between 95 and 110 kg and about six months old in the two biggest Vojvodian slaughterhouses according to routine procedure. Liver and kidney were conventionally chilled for 24 h in a chiller at 2-4°C. The samples for chemical analysis (approximately 250 g) taken after the homogenization of the whole liver and both kidney, were vacuum packaged in polyethylene bags and stored at –40°C until analysis.

Analytical methods and quality control

The cadmium (Cd) content was determined after dry ashing mineralization (Tomović et al., 2011; Gorsuch, 1970). Cd was measured in the ash solution by flame atomic absorption spectroscopy according to the manufacturer's instructions (Varian Spectra AA 10, Varian Techtron Pty Limited, Mulgvare Victoria, Australia, 1989).
The analysis of the certified reference material (ERM - CE278, Mussel tissue, IRMM, Geel, Belgium) was used for analytical quality control programme. The results of the analytical quality control programme are presented in Table 1. All analyses were performed in duplicate.

Table 1.The results of the analytical quality control programme (n = 8) used in the determination of the cadmium in liver and kidney

Element	Cd
Certified concentration (mg/kg)	0.348 ± 0.007
Recovery (%)	98.3 ± 6.22
Limit of detection (mg/kg)	0.050
Limit of quantitation (mg/kg)	0.075

Statistical analysis

All data are presented as average, standard deviation (SD) and range (Min, Max). Independent t-test and analysis of variance (one-way ANOVA) were used to test the hypothesis about differences between two or more average values. The software package STATISTICA (2008) was used for analysis.

RESULTS AND DISCUSSION

Average concentrations, standard deviations and ranges of cadmium in the liver and kidney tissue samples from five different purebred pigs are presented in Table 2. Only 3.75% of liver samples were below, while all kidney samples were above the detection limit. The minimum Cd content found in liver samples was greater than half the detection limit; therefore, all measured values were used for calculating the average (Miranda et al. 2001).

Table 2.Cadmium levels (mg/kg wet weight) in the liver and kidney of various purebred pigs from Vojvodina

	Purebred	LW	L	D	H	P	F value	*P* value[1]	Total
Liver	X	0.138	0.124	0.133	0.146	0.124	1.191	0.315	0.133
	Sd	0.060	0.072	0.050	0.059	0.049			0.059
	Min	0.052	0.029	0.031	0.057	0.039			0.029
	Max	0.268	0.270	0.252	0.261	0.227			0.270
Kidney	X	0.361	0.412	0.398	0.366	0.367	0.867	0.484	0.381
	Sd	0.121	0.249	0.184	0.117	0.137			0.169
	Min	0.168	0.173	0.178	0.203	0.180			0.168
	Max	0.654	1.160	1.060	0.649	1.012			1.160
	F value	131.015	59.194	93.136	136.446	133.328			461.650
	P value[2]	0.000	0.000	0.000	0.000	0.000			0.000

[1] *indicates significant difference between purebred within raw*

[2] *indicates significant difference between liver and kidney tissue within column*

The order of the purebred pigs according to average cadmium content in the liver samples in mg/kg was: H (0.057–0.261, on average 0.146) > LW (0.052–0.268, on average 0.138) > D (0.031–0.252, on average 0.133) > L and P (0.029–0.270 and 0.039–0.227, on average 0.124). Cadmium levels found in the present study did not differ significantly (F = 1.191; *P* = 0.315) among liver tissue for the different purebred pigs (Table 2). Individual cadmium concentrations (maximum/minimum quotient) in liver tissues differed between animals with an average of 9.3. Average wet weight level for cadmium in liver from pigs in Vojvodina was 0.133 mg/kg. The maximum cadmium concentration found (0.270 mg/kg) in the present study was below maximum level (0.5 mg/kg) set by EU and Serbian legislation (Commission Regulation (EC) No 1881/2006; Serbian Regulations 1992a, 1992b, 2002). Overall, 94.2% of liver samples had cadmium levels below half of the maximum level.

The order of the purebred pigs according to average cadmium content in the kidney samples in mg/kg was: L (0.173–1.160, on average 0.412) > D (0.178–1.060, on average 0.398) > P (0.180–1.012, on average 0.367) > H (0.203–0.649, on average 0.366) > LW (0.168–0.654, on average 0.361). Cadmium levels found in the present study did not differ significantly (F = 0.867; *P* = 0.484) among kidney tissue for the different purebred pigs (Table 2). Individual cadmium concentrations (maximum/minimum quotient) in kidney tissues differed between animals with an average of 6.9. Average wet weight level for cadmium in kidney from pigs in

Vojvodina was 0.381 mg/kg. The maximum cadmium concentration found in kidney (1.160 mg/kg) in the present study was above the maximum level (1 mg/kg) set by EU and Serbian legislation (Commission Regulation (EC) No 1881/2006; Serbian Regulations 1992a, 1992b, 2002). However, only 5 kidney samples (2.1%) slightly exceeded the tolerance level. Even 86.3% of kidney samples had cadmium levels below half of maximum level. Average cadmium concentrations in kidney were significantly higher ($F = 461.650$; $P = 0.000$) than those in liver.

The average cadmium concentrations in the pig liver and kidney from Vojvodina were in the higher range than those reported for pig liver and kidney in Spain (López-Alonso et al., 2007), Sweden (Lindén et al., 2003; Lindén et al., 2001; Lindén et al., 1999; Grawé et al., 1997; Jorhem et al., 1991), Czech Republic (Ulrich et al., 2001), Finland (Tahvonen and Kumpulainen, 1994; Niemi et al, 1991), Poland (Falandysz, 1993) and The Netherlands (Vos et al.,1986). On the contrary, the cadmium levels of liver and kidney from pigs in Croatia (Sapunar-Postružnik et al., 2001), a neighbour country of Serbia, were far above the levels found in this study, i.e. several times higher than the maximum set level. According to many authors (Andrée et al., 2010; Oskarsson et al., 2004; Lindén et al. 2003; Lindén et al., 2001; Sapunar-Postružnik et al., 2001; Lindén et al., 1999; Grawé et al., 1997; Sharma et al., 1982) the cadmium concentrations in animal tissues, especially liver and kidney, are strongly related with the cadmium levels in feedstuffs.

CONCLUSION

The results of the present study show that the levels of cadmium in the liver and kidney were not influenced by purebred. However, the variations in cadmium levels in analysed liver and kidney tissues indicate cadmium availability in the local agricultural environment in Vojvodina.

ACKNOWLEDGEMENTS

Research was financially supported by the Ministry of Science and Technological Development, Republic of Serbia, project TR31032.

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EFFECT OF ALUMINUM SALTS, COPPER SALTS AND POLYELECTROLYTES ON CHARGE NEUTRALIZATION OF PECTIN MACROMOLECULES

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ABSTRACT: In sugar industry, there is a problem of the presence of undesirable macromolecules compounds such as pectins and proteins in sugar beet juice. Separation of these compounds is done mostly by compounds with calcium ion. Affinity of calcium binding with undesirable macromolecules from sugar beet juice is not significant. The aim of this study is application of alternative coagulants with divalent and trivalent cations, $CuSO_4$ and $Al_2(SO_4)_3$ with or without the addition of polyelectrolyte which cause the process of charge neutralization macromolecules followed by polymeric bridging effect. Mechanism of discharge of macromolecules compounds using a model of double electric layer are suggested. Volume 50 cm^3 model solution of pectin (0.1 % w/w) was treated with seven volumes of $CuSO_4$ solution, ranging of 0.47 - 3.29 cm^3 and seven volumes of $Al_2(SO_4)_3$ solution, ranging of 0.59 - 4.15 cm^3. Optimal quantities of the applied coagulants, determined by measuring the Zeta potential values, where estimated as follows: 0.24 % (w/w) $CuSO_4$ and 0.45 % (w/w) $Al_2(SO_4)_3$ & Na_2CO_3 on sugar beet. This is much lower than 1 – 3 % (w/w) CaO commonly used in sugar production. Adding different concentrations of cationic polyelectrolytes in these solutions, the highest efficiency of clarification was noticed by applying cationic polyelectrolytes of concentration 3 mg/dm^3. It was shown that this type of polyelectrolyte further reduced the value of Zeta potential. Zeta potential of sugar beet pectin macromolecules was determined by electrophoretic method.
Key words: *Zeta potential, pectin, sugar beet, metal salts, polyelectrolyte*

INTRODUCTION

Charge neutralization plays a major role in undesirable macromolecules removal in water treatment. These substances are easily coagulated using metal salts. The addition of cationic polyelectrolyte in metal salts increased undesirable substances removal due to the combined charge neutralization of the metals and polyelectrolytes (Hilal et al., 2008, Duan and Gregory, 2003, Karlovič, 2002). Charge neutralization mainly takes place through the reaction of net charge opposite ions with free functional groups on the colloid surface reducing the overall net surface charge.

In sugar industry, separation of nonsaccharose compounds in the sugar beet juice, is done mostly by compounds with calcium ion. Double electric layer which surrounds all colloidal particles in solution, such as sugar beet juice, is consisted of two layers, the adsorbed and diffuse layer. The difference in potential between these layers is called electrokinetic or Zeta potential. Zeta potential is the main parameter in electrokinetic measurements, since this is a measurable value (Delgado et al., 2007, Kuljanin et al., 2008). Pectins in sugar beet juice are anionic charged colloids. Zeta potential of pectin is negative and it is strong enough to impair the coagulation and agglomeration. Addition the oppositely charged polyvalent cations can decrease the Zeta potential to near zero, causing the pectin macromolecules to discharge. In this case system's stability is lost, which leads to the coagulation and precipitation of agglomerated pectin substances (Lević et al., 2007, Kartel et al., 1999, Schneider et al., 2011). The binding affinity of divalent cations with pectins of botanical origin follows the selectivity order (Garnier et al., 1994 and Dronnet et al., 1996):

$$Cu^{2+} \sim Pb^{2+} >> Zn^{2+} > Cd^{2+} \sim Ni^{2+} > Ca^{2+} > Mg^{2+}$$

Such a diversity in the binding affinity of metal ions could be explained by two types of bindings: electrostatic binding of Ca^{2+}, Mg^{2+}, Zn^{2+} ions and formation of surface complexes by Cu^{2+} and Pb^{2+} ions. In accordance with this selectivity order, it is clearly that applying of Cu^{2+} ions will be much efficient in sugar beet juice clarification in relation to classical process by compounds with Ca^{2+} ions (Garnier et al., 1994, Wiedemer et al., 2000, Kuljanin et al., 2010).
Affinity of binding of threevalent cations with pectin macromolecules was not investigate. However, there are many studies of applying of Al^{3+} ions, especially in form of $Al_2(SO_4)_3$ in water treatment (Duan and Gregory 2003, Pattabi et al., 2000). The overall ability of pectin macromolecules to form complexes with polyvalent cations depends primarily on the degree of esterification and degree of polymerization of pectin (Pellerin et al., 1998, Dronnet et al., 1996).
Another mechanism that causes the coagulation and precipitation of the undesirable macromolecules are interparticle bridging. Interparticle bridging occur using high molecular weight polyelectrolytes where colloids are adsorbed into the polymers branches or share ions directly to form ionic bridges. (Hilal et al., 2008, Pattabi et al., 2000, Fellows et al., 2000).
The aim of the study presented in this paper was to investigate the effects of hydrolyzing metal salts ($CuSO_4$ and $Al_2(SO_4)_3$) and polyelectrolyte concentration on pectin macromolecules removal from sugar beet juice. The efficiency of ion binding, with and without addition of cationic polyelectrolyte, was determined according to electrophoretic method by measuring Zeta potential.

MATERIAL AND METHODS

Pectin preparate was extracted from cossettes obtained in the industrial processing of sugar beet (factory Žabalj). The metal salts, $CuSO_4$ and $Al_2(SO_4)_3$ in crystal hydrate form, were used for preparation the studied solutions with de-ionized water. The pH of solutions with $Al_2(SO_4)_3$ was regulated at value 7 before each experiment, using equivalent amount Na_2CO_3. High purity cationic polyelectrolyte with ammonium group (MAGNAFLOC LT-24) was purchased from Low Moor, Bradford. It has an average molecular weight ~ 100 000 kg/kmol and 100 % cationic charge density.
Extraction of pectin was conducted at pH 3.5 and 85°C during 2.5 h. The pectin preparation was precipitated from the extract with 70% ethanol solution. Basic parameters of the pectin preparation were determined according to standard methods of AOAC (Methods of Analysis of Official Analytical Chemists, 2000). Mean molar mass of pectin preparation was determined according to refractrometric and spectrophotometric method (Kar and Arslan, 1999). Degree of esterification (DE) was calculated using equivalents of free (X) and esterified carboxy groups (Y) (Fares, et al., 2003; Kuljanin et al., 2010).
In the first stage of the experiment, a series of coagulant concentrations in the range of 30, 60, 90, 120, 150, 180 and 210 mg/dm^3 was prepared by adding the necessary volumes of the solution to 50 cm^3 of 0.1 % (w/w) pectin solution. To accomplish the desired solution alkalinity (pH 7), Na_2CO_3 was added to $Al_2(SO_4)_3$ (mass ratio of Na_2CO_3 to $Al_2(SO_4)_3$ was 1 : 1.07, calculated on pure $Al_2(SO_4)_3$). After the coagulant was added to the tested preparations, pH was adjusted and the solution was stirred for 30 min on a high-speed magnetic stirrer (500 rpm). Then, the solution was stirred for another 5 min at low speed and left to rest another 5 min. In the second stage of the experiment, basic solution of flocculants was prepared by dissolving 0.5 g of flocculants in 100 cm^3 of distilled water and left overnight to swell at room temperature. In experiment was used operating solution of 0.05 % (w/w). From this solution, flocculants concentrations in the range of 1, 3 and 5 mg/dm^3 was prepared. Pectin solutions with coagulants and flocculants before Zeta potential measurements, was manually and slowly stirred for 5 min and left to rest another 5 min.
Zeta potential was determined by electrophoretic method using a commercial apparatus ZETA-METER ZM 77 (Riddick, 1975). An average value of 3 readings was used to derive the Zeta potential of colloidal particles in the tested solutions using a diagram based on the *Helmoltz-Smoluchowski* equation for electrophoretic mobility of colloidal particles. Experiments were conducted at 6-fold magnitude and voltage adjusted at 200 V.

Immediately before Zeta potential measurements, solution temperatures were measured. Zeta potential was read from the diagram and multiplied by a correction factor for a given temperature.

RESULTS AND DISCUSSION

Results related to the composition of pectin preparation are given in Table 1. The content of galacturonic acid (degree of purity) in the tested preparation is in agreement with the mean content of pectin found in raw sugar beet juices from diffuser reported in literature. The obtained preparation was low esterified pectin (DE < 50) and the molar mass was determined by measuring 5 different concentrations of pectin (Kar and Arslan, 1999).

Table 1. Basic physico-chemical composition of pectin preparation

Solid content, SC (g/100g)	Equivalent of free COOH groups, $X \cdot 10^5$	Equivalent of ester. COOH groups $Y \cdot 10^5$	Content of galacturonic acid (%)	Degree of esterific. DE	Mean molar mass, M_{Wsr} (kg/kmol)
80.35	24.58	16.05	72.24	39.50	87 720

Changes of mean values of Zeta potential (mV) of the tested preparation after adding various quantities of coagulants $CuSO_4$ and $Al_2(SO_4)_3$ in the form of pure salts (mg/dm^3) are shown in Figure 1. Changes of mean values of Zeta potential after the addition of coagulants and cationic flocculants are shown in Figures 2 and 3.

In all experiments, charge inversion of Zeta potential from negative to positive was observed within the whole series of tested coagulants concentrations. Total net charge of Cu^{2+} and Al^{3+} ions (including H^+ ions in the solution) increased in magnitude in comparison to the magnitude of negative charge on the surface of pectin macromolecule. This proves that, besides ionic exchange and lowering of surface potential by charge neutralization, surface complexation of Cu^{2+} and Al^{3+} ions with COO^- groups of pectin macromolecules was occurred. The results indicate that heavy metal ions Cu^{2+} link stronger to polygalacturonic chains of pectin macromolecules than lighter cations Al^{3+}. According to the *Schulze-Hardy* rule, ions with high valence like Al^{3+} should be able to decrease Zeta potential to zero point at much lower concentration. However, from the results presented on Figure 1, it is obvious that in the tested preparation, less amount of $CuSO_4$ (82.0 mg/dm^3) compared to $Al_2(SO_4)_3$ (105 mg/dm^3) was required for lowering Zeta potential to zero point. This can be explained by higher binding ability of these ions considering that Cu^{2+} ions are first-ranged in the previously given selectivity order. It is possible that other mechanisms also take part:

- since Cu^{2+} ions have higher density than Al^{3+} ions, their kinetic energy is higher enabling their easier location in the adsorbed part of double electric layer, neutralising the surface charge of pectin macromolecules.
- Cu^{2+} ions are less hydrated due to larger ionic diameter in comparison to Al^{3+} ions. Less hydrated Cu^{2+} ions affects the velocity of their distribution into the diffuse layer of the double electric layer and consequently, into the adsorbed layer on the macromolecule surface.

If the quantity of $CuSO_4$ is recalculated at the industrial level, 0.24 % (w/w) on sugar beet will be obtained. The combination of $Al_2(SO_4)_3$ & Na_2CO_3 is less efficient. If the quantity of $Al_2(SO_4)_3$ & Na_2CO_3 is recalculated at the industrial level, 0.45 % (w/w) on sugar beet will be obtained. This is much lower than 1 – 3 % (w/w) CaO commonly used in sugar production.

Results of experiments carried out with the cationic polyelectrolytes, show a slight deviation change in Zeta potential using concentrations of 3 and 5 mg/dm^3 (Figure 2 and 3). The addition of cationic flocculants concentration of 3 mg/dm^3, the required amount of coagulant $Al_2(SO_4)_3$ to decrease the Zeta potential to zero point is less and amounts to 95 mg/dm^3 (Figure 3) while the amount of coagulant $CuSO_4$ even lower, at around 70 mg/dm^3 (Figure 2). Though not extensively reported in literatures, it is highly possible that cationic polyelectrolyte provides additional charge neutralization associated with mechanism of interparticle bridging.

Interparticle bridging includes that the pectic macromolecules are adsorbed into the high molecular weight polyelectrolytes branches.

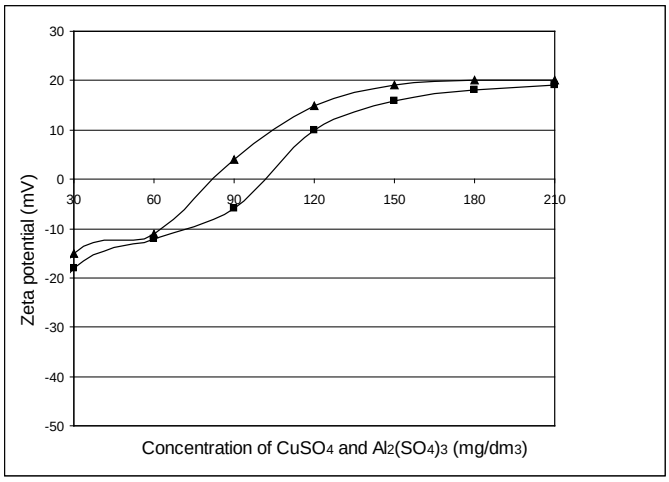

Figure 1. Dependence of the Zeta potential of pectin solution on the $CuSO_4$ and $Al_2(SO_4)_3$ concentration: ▲- $CuSO_4$ ■ - $Al_2(SO_4)_3$

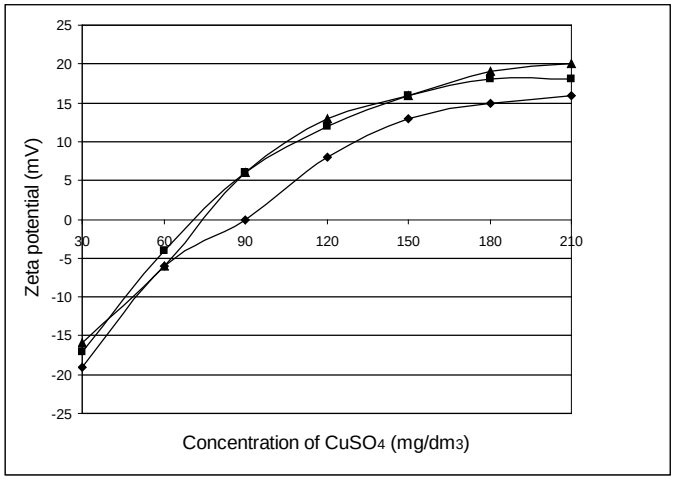

Figure 2. Dependence of the Zeta potential of pectin solution on the $CuSO_4$ concentration using three concentration of cationic flocculants: ♦ - 1 mg/dm^3; ■ - 3 mg/dm^3; ▲– 5 mg/dm^3

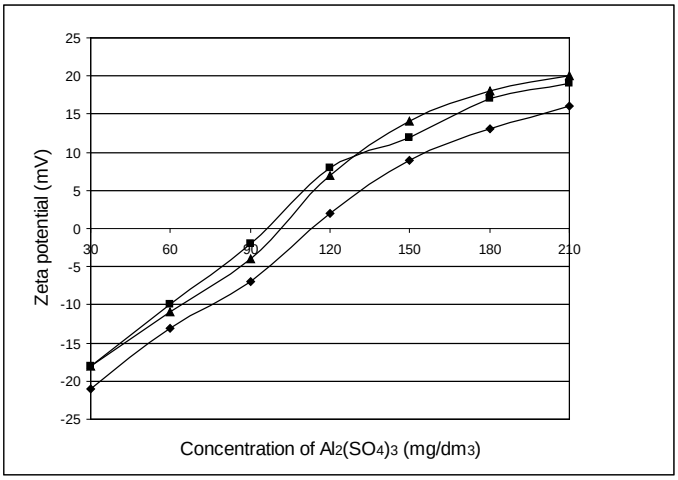

Figure 3. Dependence of the Zeta potential of pectin solution on the $Al_2(SO_4)_3$ concentration using three concentration of cationic flocculants: ♦ - 1 mg/dm^3; ■ - 3 mg/dm^3; ▲– 5 mg/dm^3

CONCLUSIONS

The influence of metal ions and cationic polyelectrolyte on Zeta potential of sugar beet pectin solution has been investigated. Hydrolyzable metal salts, $CuSO_4$, $Al_2(SO_4)_3$ and cationic polyelectrolyte all had a pronounced effect on the magnitude of the particle Zeta potential, which reflected in coagulation and flocculation performance. Cu^{2+} ions have larger influence on surface charge of pectin macromolecules and consequently higher destabilizing effect in comparison with Al^{3+} ions.
Cationic polyelectrolyte (MAGNAFLOC LT-24, concentration of 3 mg/dm^3) in combination with these metal salts, produced better charge neutralization and inter-particle bridging of pectin macromolecules in comparison with metal salts without polyelectrolytes.
Besides the tested ions and polyelectrolyte, for successful coagulation of pectin macromolecules, numerous other factors must be investigated such as kind of ions and polyelectrolytes, mixing effect and effect of mixture of different ions.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support from the Ministry of Education and Science of the Republic of Serbia (Project III 46005).

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FATTY ACID COMPOSITION OF ROSEMARY (*ROSMARINUS OFFICINALIS* L.) LEAVES

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ABSTRACT: Since ancient times, aromatic herbs and spices have been added to different types of foods to improve the flavour and sensory properties.Rosemary (*Rosmarinus officinalis* L.) is a perennial herb that belongs to Lamiaceae family. It is used as a food flavouring agent and is also well known medicinally for its powerful antimutagenic, antibacterial and chemopreventive properties. Additionally, it has been demonstrated that the plant exhibits antioxidant activity. The leaves of rosemary as well as other wild edible plants used for culinary purposes give low yields of oil, but this oil is a rich source of essential fatty acids, such as α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6). Linolenic acid and fatty acids of n-3 and n-6 series play an important role in the modulation of human metabolism. In this study, the qualitative and quantitative composition of leaf lipids of rosemary (*Rosmarinus officinalis* L.) grown in Serbia has been investigated. Gas chromatography – mass spectrometric analyses showed major fatty acids to be α-linolenic, linoleic and palmitic acids of which contents were within the ranges of 32.4-42.0; 21.3-27.9 and 12.5-17.1% of the total fatty acids, respectively. Results showed that rosemary is rich source of essential fatty acids (18:3n-3 and 18:2n-6), with favourable n-6/n-3 ratio.
Key words: *fatty acid, rosemary, gas chromatography, mass spectrometry*

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is a very important medicinal and aromatic plant, which belongs to the Lamiaceae family and has been cultivated for a long time. Anthropologists and archaeologists have found evidence that rosemary branchs with their leaves were used as medicinal, culinary and cosmetic virtues in the ancient Egypt, Mesopotamia, China and India (Stefanovits-Bányai et al., 2003). Nowadays, rosemary is one of the most appreciated sources of natural bioactive compounds which are of special interest in the functional food industry (Borrás Linares et al., 2011). In fact, this plant exerts a great number of pharmacological activities, such as antimicrobial (Santoyo et al., 2005; Bozin et al., 2007), antiviral (Auroma et al., 1996), antitumor (Huang et al., 2005), anti-inflammatory (Altinier et al., 2007) and antioxidant (Pérez-Fons et al., 2010; Bozin et al., 2007) activities.
Rosemary is an evergreen branched bushy shrub, attaining a height of about one meter with upright stems and dark green leaves which are small and with edges turned over backward. Underneath these rolled edges are little glands containing aromatic oils. The leaves of rosemary as well as other wild edible plants used for culinary purposes give low yields of oil, but are rich sources of essential fatty acids, such as α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA,18:2n-6). Both LA and ALA acid are metabolized to longer chain polyunsaturated fatty acids with 20 and 22 carbon atoms. Omega 6- and omega 3-polyunsaturated fatty acids (PUFA) have specific physiological functions, they are necessary for proper growth and development and are therefore of nutritional importance (Trautwein, 2001). Omega-3 fatty acids play an important role in modulating human metabolism and have beneficial effects against cardiovascular disease and cancer, two of the major diseases of the 21st century. Hence omega-3 PUFA has been widely accepted as one of the cornerstones of healthy lifestyle and nutrition.

MATERIAL AND METHODS

Samples of rosemary (*Rosmarinus officinalis* L.) were collected in October 2010 from three different locations in Vojvodina (Northern part of Serbia). For lipid extraction and FA analysis, only the leaves were used, which were removed from the stems. The moisture content was determined drying to the constant mass by the oven method (105°C for 3 h). Total lipids from leaves were extracted according to the method of Soxhlet using petroleum ether as solvent and further analysis were done by gas chromatograph equipped with mass spectrometer detector (GC-MS).

Preparation of fatty acids methyl esters

Fatty acid methyl esters were prepared using a fast method (Kravić et al. 2006), as follows: approximately 60 mg of extracted fat was put into a test tube and dissolved in 2.4 ml of hexane. An aliquot (0.6 ml) of 2 mol/dm^3 methanolic KOH solution was added. The tube was capped and vigorously shaken for 20 s and allowed to boil one minute in water bath at 70°C. After 20 s of shaking 1.2 ml of 1 mol/dm^3 HCl was added and gently stirred. After phase separation 3 ml of hexane was added, and the upper phase containing the fatty acid methyl esters were decanted and dissolved in hexane to 5 ml. Aliquot of 1 µl was taken for GC-MS analysis.

Gas chromatography-mass spectrometry analysis

The analysis of fatty acid methyl esters were performed on a Hewlett-Packard (HP) 5890 gas chromatograph coupled with a HP 5971A mass spectrometer detector. A fused silica capillary column SP-2560 (100 m × 0.25 mm i.d., 0.20 µm film thickness) was used. The inlet temperature was 230°C with a split ratio 1:20. Carrier gas was helium with constant flow rate of 0.58 cm^3 min^{-1}. The initial temperature of 100°C was held for 5 min and increased by 6°C/min to an end temperature of 240°C. The mass spectrometer was operated in the electron ionization mode with quadruple temperature of 180°C. Data acquisition was carried out in the scan mode (range 50 - 400 m/z). A multistandard from Supelco (Cat. No. 47885-U, Bellefonte, PA, USA) containing the methyl esters of 37 fatty acids was used to confirm the retention times and mass spectra for peak identification, as well as to confirm that the peak areas reflected the actual composition of these mixtures.

RESULTS AND DISCUSSION

Samples locality and moisture content are given in Table 1. Table 2 shows the fatty acid composition of rosemary leaves. Fatty acid contents are expressed as weight percentages of total fatty acid methyl esters. Values are presented as the mean ± standard deviation (SD) of three replicates.

To the best of our knowledge, no earlier studies were reported in literature regarding the fatty acids composition of rosemary leaves. Thus, we reported that α-linolenic was the major fatty acid, followed by linoleic and palmitic acids, with mean values of 38.5, 24.8 and 15.0%, respectively. These three fatty acids represent 78% of rosemary total fatty acids. Stearic and oleic acid were presented at moderate level, presenting together 15%. Linoleic and α-linolenic acids are two essential fatty acids that humans and other mammals must ingest for good health because their body requires them for various biological processes but cannot biosynthesize them from other food components (Bettaieb et al., 2011). The contents of fatty acids are in agreement with another report for other edible plant species (Guil-Guerrero and Rodríguez-García, 1999) in respect to the predominant fatty acids.

Table 1. Sample (*Rosmarinus officinalis* L.) locality and moisture content of rosemary leaves

	Sample locality	**Moisture content** (g/100 g)
Sample 1	Novi Sad	55.42
Sample 2	Rumenka	52.21
Sample 3	Irig	54.34

Table 2. Fatty acid centesimal composition of total lipids from rosemary leaves

fatty acid	**Sample 1**	**Sample 2**	**Sample 3**
12:0	0.2 ± 0.01	ND	ND
14:0	1.1 ± 0.03	ND	ND
16:0	12.5 ± 0.12	15.4 ± 0.23	17.1 ± 0.41
16:1	0.5 ± 0.04	ND	ND
17:0	0.3 ± 0.02	ND	ND
17:1	1.2 ± 0.07	ND	ND
18:0	5.2 ± 0.03	5.7 ± 0.31	5.8 ± 0.31
18:1	10.1 ± 0.01	9.8 ± 0.13	9.1 ± 0.13
18:2n-6	21.3 ±0.21	27.9 ± 0.69	25.2 ± 0.15
20:0	2.3 ± 0.06	ND	5.4 ± 0.15
20:1	0.9 ± 0.07	ND	ND
18:3n-3	42.0 ± 0.08	41.1 ± 0.17	32.4 ± 0.92
22:0	1.7 ± 0.12	ND	2.7 ± 0.11
24:0	0.8 ± 0.06	ND	2.3 ± 0.13
SFA	24.1	21.1	33.3
MUFA	12.6	9.8	9.1
PUFA	63.3	69.1	57.6
∑ n-6	21.3	27.9	25.2
∑ n-3	42.0	41.1	32.4
n-6/n-3	0.5	0.7	0.8

Results are given as mean ± standard deviation (n = 3); ND – not detected; SFA - saturated fatty acid; MUFA - monounsaturated fatty acids; PUFA – polyunsaturated fatty acids

In the last decades, it is widely accepted that an excessive intake of saturated fatty acids and/or a low intake of omega-3 polyunsaturated fatty acids may lead to series of severe metabolic disorders. As can be seen from the obtained results rosemary leaves were characterised by a high proportion of polyunsaturated fatty acids (mean value 63.3%) versus 26.2% of saturated and 10.5% of monounsaturated ones. Moreover, examined rosemary samples have had favourable n-6/n-3 ratio, which were between 0.5 and 0.8. An n-6/n-3 fatty acid ratio of 5:1 or less is desired, as suggested by nutrition experts (Rubio-Rodríguez et al., 2010). Reducing the ratio of n-6 to n-3 polyunsaturated fatty acids to 4:1 has been found to reduce total mortality by 70% in secondary prevention of cardiovascular disease; a ratio of 5:1 is beneficial for asthma; 2–3:1 reduces rheumatoid arthritis inflammation and a ratio of 2.5:1 reduces colorectal cancer cell proliferation (McManus et al., 2011). The plant cell membranes are dynamic in behaviour, with a lipid composition depending on the age of the plants and environmental factors such as temperature, drought, salinity levels and the other. No negligible difference in palmitic, linoleic, linolenic and arahidic acid contents, among rosemary samples probably were results of different cultural conditions.

CONCLUSIONS

The fatty acid composition of rosemary (*Rosmarinus officinalis* L.) leaf lipids were determined by gas chromatography-mass spectrometry. Obtained results showed that the rosemary is rich source of polyunsaturated essential fatty acids (linoleic and α-linolenic), with favourable n-6/n-3 ratio. These indicated that fatty acid composition improved other well known nutritional values of rosemary.

ACKNOWLEDGEMENTS

This paper is a result of bilateral cooperation between Republic of Serbia and Kingdom of Spain, supported by the Ministry of Education and Science of the Republic of Serbia and Spanish Ministry of Education and Science (PRI-AIBSE-2011-1228).

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THE EFFECT OF HULLS ON *ALTERNARIA* SPP. INFECTION OF SPELT WHEAT KERNELS AND IMPACT OF LEVEL OF INFESTATION ON LENGTH AND WEIGHT OF SPIKES

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ABSTRACT: There is a growing interest in spelt wheat (*Triticum aestivum* ssp. *spelta* L.) due to its low input requirements and valuable nutritional properties. Compared to common wheat, spelt might be more resistant to fungal diseases and perform better in ecologically friendly farming systems. Genus *Alternaria* includes pathogenic and saprophytic species causing economic losses in food production. Many *Alternaria* spp. are allergens and mycotoxin producers harmful for human and animal health. The aim of this work was to investigate the effect of hulls in protection of spelt kernel against penetration of *Alternaria* spp. Additionally, impact of *Alternaria* spp. infestation on length and weight of spike was assessed. Field experiment was carried out in the 2010/2011 season in the region of Vojvodina on three spelt wheat genotypes. Inoculation was performed with an aqueous suspension of *A.alternata* conidia and two isolates of *A.tenuissima* obtained from 14-day old cultures grown on a PDA medium. Spikes treated with fungicide and distilled water treatments were used as two control objects. It was found a significant difference of infection level between hulled (92.3-96.6%) and dehulled (23.3-88.0%) spelt samples, with exception of *A.alternata* treatment. Yield components, length of spike and weight of spike have shown significant difference between fungicide treatment (16.2 cm; 1.82g) and infected kernels by *Alternaria* spp. (10.7cm; 1.03g) respectively. According to obtained results it could be concluded that hulls protect spelt kernels against fungal colonization and therefore increase the eligibility of spelt wheat under unfavorable conditions.
Key words: *Alternaria spp., spelt, hulls, length and weight of spike*

INTRODUCTION

Spelt wheat (*Triticum aestivum* ssp. *spelta*.) is a neglected subspecies of common wheat, which has been prompted recently by the development of organic farming (Konvalina et al., 2010). It had been grown in Europe (mainly in Germany, Switzerland and Austria) for hundreds of years when it was largely replaced by bread wheat (Dark and Gent, 2001). There is a growing interest on the world market for such alternative crop due to its high nutritive and dietetic values (Gomez-Becerra et al., 2010). Stable yielding ability under low input conditions and high tolerance to environmental stresses, consider spelt economically important culture suitable to organic farming system (Rüegger and Winzelerund, 1993; Lacko-Bartošová et al., 2011).

Compared with common wheat, spelt carries high levels of resistance to several fungal pathogens (Kema, 1992), which is probably associated with its morphological properties. Hulled grain cereals are generally more resistant to fungal diseases (Konvalina et al., 2010; Mouldry et al., 2011; Khatibi et al., 2012). Previous studies on spelt wheat indicate that the hulls provided an advantage to the seed germination (Ruegger et al., 1990) and act as barriers against soil borne pathogens (Riesen et al., 1986). Protective effect of hulls on fungal colonization and their metabolites has been reported mostly for *Fusarium* spp. (Moludry et al. 2009; Suchowilska et al., 2010; Wiwart et al., 2009; Bodroža et al., 2010),

Penicillium spp. (Elmholt, 2004) and *Puccinia* spp. (Kema, 1992; Konvalina et al., 2010) etc. There is, however, extremely limiting information on *Alternaria* species concerning hulled grain varieties.

The genus *Alternaria* is widely spread and includes plant pathogenic and saprophytic species that may affect crops in the field or can cause harvest and postharvest decay. Due to their growth even at low temperature, *Alternaria* spp. are also responsible for spoilage of food during refrigerated transport and storage (Ostry, 2008). Beside losses in production and processing, many species of this mycobiota are able to produce toxicological metabolites, which are harmful for human and animal health, (Logrieco et al., 2009). Some *Alternaria* toxins might have even the cancerogenic effect (Liu et al., 1992). *Alternaria* spores, disseminated mainly by air currents, can influence allergen reactions and respiratory problems as well (Corden et al., 2003; Kilic et al., 2010; Pavon et al., 2010). Recent studies reported that fungi of the genus *Alternaria* dominates on wheat kernels (EFSA, 2011) and are frequently found on spelt wheat (Kurowski and Wysocka, 2009). The aim of this work was to investigate the protection effect of hulls of spelt kernels to *Alternaria* spp. disease with addition to evaluation of influence of fungal infection to yield components such as length and weight of spike.

MATERIAL AND METHODS

Field experiment was carried out in the 2010/2011 season in the region of Vojvodina, north Serbia on three spelt wheat genotypes.

Fungus culture and inoculation

The isolates of *A.alternata* and two isolates of different species *A.tenuissima* were multiplied on petri-plates containing potato dextrose agar (PDA) at room temperature for 14 days. For the purpose of inoculation a conidial suspension was made in water and sprayed on the plants with a hand atomizer. Before spraying, flasks were shaken vigorously and 1 mL suspension was poured in to a haemocytometer and the number of spores counted. Concentration of *A.alternata* conidia was $0{,}2475 \times 10^6$ infective particles/mL and two isolates of *A.tenuissima* had concentrations of $0{,}605 \times 10^6$ and $0{,}497 \times 10^6$ infective particles/mL. At the full flowering stage inoculation was performed with 600 mL aqueous suspension of fungal isolates. Inoculated spikes were immediately covered with polyethylene bags for 24 h. Spikes treated with fungicide and water treatments were used as two control objects. In the full ripeness stage spikes from each plot were cut by hands and used for next analysis.

Yield characteristics

These have been based on the methodology of the evaluation of length and weight of spelt wheat spikes. Data on length and weight of spike were recorded in ten repetition on randomly selected samples.

Preparation of plant material

Spelt kernels, from each treatment, were dehulled using laboratory thresher (model MDF1, RePietro, Gaggiano, Italy). Dehulled and hulled kernels were kept separately in dark and dry place before assessing the level of contamination.

Percent of kernel infection

According to the method proposed by Pitt and Hocking (1985) 100 dehulled and hulled spelt kernels were randomly selected from each treatment. The samples were desinfected with 0,4% NaClO, rinsed with water for 2 minutes and placed on petri plates in four repetitions (25 kernels per plate). Incubation was conducted at room temperature and after six days intensity of infection was assessed.

Statistical analysis
Statistica 8.0 Software was used for statistical data processing using one-way ANOVA. The comparison of mean values were performed by the Fisher- test. A value of $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

The obtained results showed a high amount of *Alternaria* isolates in inoculated samples, which is in agreement with Kurowski and Wysocka (2009), where in natural occurrence survey in different farming systems, predominant fungal species was *A.alternata*. In evaluation of level of infection, genomic diversity of spelt wheat did not take in consideration, inasmuch values obtained from different genotypes implied as replication values. It was found a significant difference of infection level between hulled (92.3-96.6%) and dehulled (23.3-88.0%) spelt kernels, with exception of *A.alternata* treatment (Figure 1). That could be explained with higher aggressiveness of *A.alternata* species. These results confirm previous researches where dehulled kernels were 50% less contaminated with *Alternaria* spp. compared to hulled samples (Vučković et al., 2011). The highest level of infection was determined on hulled kernels inoculated with *A.tenuissima* (1) (96.6%), while the lowest was on dehulled kernels treated with fungicide (23.3%).

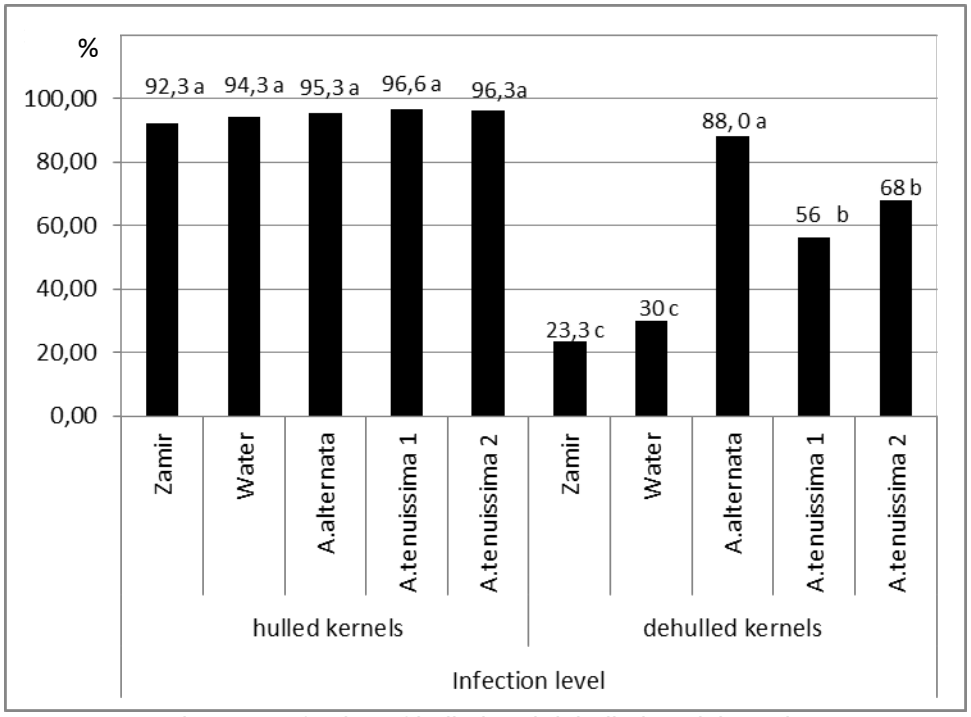

Figure 1. Infection of hulled and dehulled spelt kernels
Means followed by the same letter are not significantly different at P=0.05 (Fisher- test);

Yield components, length and weight of spike, have shown a significant difference between fungicide treatment and infected kernels by *Alternaria* spp. in general, which is presented in Tables 1 and 2. The longest spike was detected in fungicide treatment at genotype 3 (16.15 cm), while the lowest length was at inoculation with *A. tenuissima* (1) at genotype 2 (10.7cm). Maximum weight of spike was in fungicide treatment at genotype 3 (1.82 g), while minimum weight has the same genotype but in *A. tenuissima* (1) treatment (1.03g). The fungal inoculation of spikes led to considerable reduction of the main yield parameters of all the tested spelt genotypes which is in line with results obtained by Suchowilska et al. (2007) in experiment with *Fusarium culmorum*. In organic farming systems, whereas synthetic fungicides are forbidden, spikes of the spelt plants were very long (11.3 cm) according to Konvalina et al. (2010), which point that spelt could have stable yield in organic production.

Table 1. Mean values of length of spikes obtained from inoculation treatments

Spelt genotype	Treatment	Length of spike (mean)
2	At(1)	10.70^{g}
1	At(2)	10.90^{fg}
1	At(1)	11.70^{efg}
3	At(2)	11.75^{efg}
1	Aa	12.00^{def}
2	At(2)	12.25^{cde}
2	Aa	12.65^{cde}
3	At(1)	12.85^{cde}
3	Aa	13.15^{bcd}
1	W	13.28^{bcd}
2	W	13.40^{bc}
3	W	13.50^{bc}
2	F	14.16^{b}
1	F	14.30^{b}
3	F	16.15^{a}

Table 2. Mean values of weight of spikes obtained from inoculation treatments

Spelt genotype	Treatment	Weight of spike (mean)
3	At(1)	1.03^{e}
2	At(1)	1.05^{e}
1	Aa	1.12^{e}
1	At(1)	1.19^{de}
1	At(2)	1.23^{cde}
3	At(2)	1.37^{bcd}
2	At(2)	1.40^{bcd}
3	Aa	1.41^{bcd}
2	Aa	1.44^{bc}
2	W	1.45^{bc}
1	W	1.47^{bc}
3	W	1.49^{b}
1	F	1.50^{b}
2	F	1.58^{ab}
3	F	1.82^{a}

Abbreviations used in table: F-fungicide treatment, W-control with water, Aa- A.alternata, At (1)-A.tenuissima (1), At (2)-A.tenuissima (2); Within column values followed by the same letter are not significantly different at P < 0.05 (Fisher- test).

CONCLUSION

The results of our research indicate that fungicide and inoculation treatments have significant impact on length and weight of spikes which were in the range of 10.7-16.5 cm and 1.03-1.82g respectively. The hulls have protective effect of spelt kernels inside from *Alternaria* spp. It should be stressed, however, that protection is partially and additional protective measures are recommended. Additionally, this pathogen deserves more attention considering its toxicological properties and further investigations of *Alternaria* toxins are expected. It could be concluded that hulling will contribute in reduction of mycobiota on spelt kernels.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III46005 “New products based on cereals and pseudocereals from organic production”, financed by the Ministry of Science and Technological Development, Republic of Serbia.

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RESEARCH OF PATULIN IN FROZEN RASPBERRIES CONTAMINATED WITH FIELD FUNGI

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ABSTRACT: Fresh fruit is susceptible to contamination with filamentous fungi during growing, harvesting, transport, storage and sales. For these reasons it is important to identify and take appropriate measures not only about microbiological contamination but also concerning possible presence of mycotoxins. Due to soft tissue structures berries are very susceptible to mold contamination of different mold genera including toxigenic ones (*Penicillium, Aspergillus and Byssochlamys*). The aim of this research was to investigate the presence of mycotoxin patulin in frozen raspberries harvested under rainy and wet conditions convenient for production of this secondary metabolite of molds in fresh raspberries. Frozen raspberry samples which were visually rated as products with intensive mold attack were taken from the most important cooling warehouses in raspberry growing region in Serbia. The method developed by Arranz et al (2005) widely represented in the EU for the determination of patulin using high-performance liquid chromatography (HPLC) with UV detection (λ = 276 nm) was used for determination of patulin in the raspberry samples. Determined contents of patulin in the samples were ranged from 1.07 to 15.33 µg/kg. Patulin content in fresh fruits as raw material for further processing is not regulated by laws in Serbia or in the European Union, but the Joint Food and Agriculture Organization-World Health Organization (WHO) Expert Committee on Food Additives has established a provisional maximum tolerable daily intake for patulin of 0.4 µg/kg of body weight per day (WHO, 1995).
Key words: *raspberry, patulin, HPLC-UV*

INTRODUCTION

Patulin is mycotoxin produced by a number of fungi (*Penicillium, Aspergillus* and *Byssochlamys*) common to fruit- and vegetable-based products. *Penicillium expansum,* the blue mold that causes soft rot of apples, pears, cherries, berries and other fruits, is one of the most common sources of patulin contamination (Dursch and Ragab, 2003; Watanabe, 2008). Patulin-producing strains have been isolated from a variety of fruits and vegetables including apples, grapes, cherries, crabapples, pears, apricots, persimmons, strawberries, nectarines, raspberries, black mulberries, white mulberries, lingon berries, peaches, plums, tomatoes, greengages, bananas, blueberries, black curants, almonds, pecans, peanuts, and hazelnuts (Moake et al., 2005).

Assessment of the health risks posed by patulin to humans is based upon a wide number of studies during the past 50-plus years that implicate a number of acute, chronic, and cellular level health effects. Results of many studies suggest that acute symptoms of patulin consumption can include agitation, convulsions, dysponea, pulmonary congestion, edema, ulceration, hyperemia, GI tract distension, intestinal hemorrhage, epithelial cell degeneration, intestinal inflammation, vomiting, and other gastrointestinal and kidney damage. Chronic health risks of patulin consumption can include neurotoxic, immunotoxic, immunosuppressive, genotoxic, teratogenic, and carcinogenic effects (Moake et al., 2005). Therefore, patulin contamination is a worldwide problem with regard to food and feed safety, and several countries have instituted patulin restrictions in food products (Doores 1983; Spott and others 1993).

In 1995, a provisional maximum tolerable daily patulin intake of 0.4 µg/kg body weight was established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1995).The

permissible limit for patulin content in apples and their products in the European Union and Serbia has been set at 50 µg/kg, in apple juice, and as juice ingredients in other beverages, has been set at 50 µg/kg, in solid apple products at 25 µg/kg, and in baby food of 10 µg/kg (Commission Regulation (EC) 1881/2006; Sl. glasnik R. Srbije 28/2011). In view of the recognized adverse effects of patulin and the need for regulatory control, monitoring of its level in food products is important to evaluate the risk associated with human consumption of these products. Therefore, a sensitive, selective, and simple method to determine the presence and contents of patulin in raspberry samples is required. Due to soft tissue structures berries are very susceptible to mold contamination of different mold genera including toxigenic ones (*Penicillium, Aspergillus and Byssochlamys*).

For Serbia raspberry is one of the most important agricultural export commodities due to significant and stable export demand for frozen raspberry. Out of total raspberries production more than 90% is dedicated for export markets. During the last years average production of raspberry in Serbia ranged around 80.000 tons, representing share of 5.5% in total fruit production. About 25% of global raspberry production originates from Serbia Serbia exports mainly frozen and only in rare cases cooled fresh raspberry *(Dimitrijević, 2009)*.

Raspberries are grown for the fresh fruit market and for commercial processing into individually quick frozen (IQF) fruit, purée, juice, or as dried fruit used in a variety of grocery products. Traditionally, raspberries were a mid-summer crop, but with new technology, cultivars, and transportation, they can now be obtained year-round. Raspberries need ample sun and water for optimal development. Raspberries thrive in well-drained soil with a pH of between 6 and 7 with ample organic matter to assist in retaining water.

As a cultivated plant in moist temperate regions, it is easy to grow and has a tendency to spread unless pruned. Based on years of statistical data we can conclude that the Serbian raspberries scale production is the leader on the world stage (from 1987 to 2009 was the second place behind Russia) (http://faostat.fao.org/site/339/default.aspx).

In Serbia the raspberry harvesting season (early and mid summer) is usually characterized with hot and dry weather but in exceptional years like 2010 in late June and early July during fruiting of raspberries abundant precipitation were recorded dailyin the areas where the crops are grown. From mid June to mid-July rainfall sum of 40% to 100% above the perennial average for the same period was recorded. Although raspberry is a plant that for successful growing and high yield requires a lot of water especially during ripening, such weather conditions result in increased health safety risks especially from the aspect of fungi development (www.hidmet.gov.rs)

The aim of this research was to investigate the presence of patulin in frozen raspberries harvested under rainy and wet conditions convenient for production of this secondary metabolite of molds in fresh raspberries.

MATERIAL AND METHODS

Samples

Five random samples of frozen raspberries harvested in 2010 were taken from cooling warehouses in raspberry growing region in Serbia around Arilje. Selection of samples was performed on the basis of their visual rating as products with intensive mold attack. Raspberries were frozen immediately after harvest and kept in frozen condition for 6 months.

Reagents

Acetonitrile, ethyl acetate and n-Hexane (HPLC grade), ethanol, acetic acid (extra pure grade), anhydrous sodium sulfate, anhydrous sodium hydrogen carbonate, perchloric acid 60% and sand (p.a.) were purchased from Merck (Darmstad, Germany). The patulin standards were obtained from Sigma-Aldrich. Water was ultra pure (Mili-Q, from Milipore, USA).

Sample preparation
The basic principle of the method developed by Arranz et al. (2005) is based on the extraction of surfactants from the sample with a mixture of ethyl acetate-hexane (60:40, v / v) in the sand, Na_2SO_4 and $NaHCO_3$, and then the extract passed through a C18 SPE column (SPE, Supelco, Bellefonte, PA). The purified extract was evaporated to dryness under stream of nitrogen and redissolved in aqueous acetic acid solution (pH 4). The redissolved samples were filtered through a membrane filter (0.45 µm) and the filtrate was transferred to an HPLC vial and analyzed on HPLC-UV.

Patulin stock solution (200 µg/ml in ethyl acetate)
Stock solution was prepared by dissolving 5 mg of patulin standard in 25 ml of ethyl acetate. This solution was stored in the freezer at -20°C.

Patulin working standard solution for calibration graph
Patulin working standard solutions were prepared from patulin calibrant solution. A series of nine standard working solutions between 0.93 and 89.16 µg/ml were used for calibration graph (R^2=0.9995).

Determination of patulin by HPLC-UV
Numerous chromatographic methods have been developed for the determination of patulin in various food products. One of the most used methods for the quantitative determination of patulin in apple-based products is method published by Arranz et al. (2005).
The aim of this work was to apply procedure for sample preparation described in Arranz method for analysis of raspberry samples and also to use different chromatographic conditions. Determination of patulin was carried out by HPLC Agilent 1200 model equipped with Agilent ultra violet detector (UV), Chemstation Software, a binary pump, a vacuum degasser and an auto sampler. The column was an Agilent RP-HPLC column Zorbax eclipse XDB-C18 (100 x 2.1 mm, particle size 1.8 µm). The mobile phase consisted of an isocratic mixture of with water/acetonitrile/perchloric acid 60% (990+10+1, v/v/v), with a flow rate of 0.3 ml/min. Ten microliters of standards and samples were injected onto the HPLC column. All determinations were performed in duplicate. The spectra were recorded at 276 nm. Identification of patulin was performed by comparing of the retention times and spectra of patulin from samples with those of the standards.

Spike recovery solution
In order to calculate the recovery spiking was performed with sample containers identified as samples with the lowest contents of patulin (sample 2). One milliliter of patulin standard (59.4 µg/kg) was added in 19.0 g defrosted raspberry puree (spiked sample 1), and it was prepared according to the instructions by Arranz et al (2005). This procedure was replayed with 1 ml patulin standard with 86.3 µg/kg patulin contents (spiked sample 2).

RESULTS AND DISCUSSION

The presence of patulin was determined in all of the examined raspberry samples. Determined contents of patulin in the samples were ranged from 1.07 to 15.33 µg/kg. The average values of patulin content in the examined raspberry samples are shown in Table 1.

Table 1. Average values of patulin content, interval (CI) and mean (CM±SD) of raspberry samples

No. samples	patulin contents (µg/kg)
1	1.30
2	1.07
3	2.42
4	15.33
5	3.33
CI	1.07 – 15.33
CM±SD	4.76 – 6.00

SD: standard deviation

Regardless of climatic conditions that prevailed during 2010, which were suitable for the development of pathogenic species of fungi on raspberries, the resulting patulin contents in the analyzed raspberry samples were significantly below the contents of patulin obtained by Demirci et al (2003) for mold contaminated raspberries from other raspberry growing region (48 to 746 µg/kg, with an average content 325.73 µg/kg). This fact can probably be explained either as a consequence of the resistance of the varieties and protection measures applied during growing of raspberries or on the basis of possibility of presence of nontoxigenic mold species in respect to patulin.
Determined precision characteristics of the modified method applied on the raspberry samples are presented in table 2).

Table 2. Method performance characteristics obtained for raspberry puree

Spiked sample	**Results**		2006/401/EC
	1	2	
RSD_r %	17.96	11.00	≤ 30
RSD_R %	9.30	10.28	≤ 40
Recovery %	75.1	92.8	50 to 120

RSD_r – relative standard deviation, calculated from results generated under repeatability conditions
RSD_R - relative standard deviation, calculated from results generated under reproducibility conditions

Based on the obtained results for RSD_r, RSD_R and Recovery it can be concluded that modified method of Arranz (2005) can be used for determination of patulin in raspberry samples.
The method showed acceptable within-laboratory precision for examined matrix, according to Commission Regulation (EC) 2006/401/EC (table 3).

Table 3. Performance criteria for patulin according to Directive 2006/401/EC for analytical methods for the determination of patulin

Level µg/kg	**Patulin**		
	RSD_r %	RSD_R %	Recovery %
< 20	≤ 30	≤ 40	50 to 120
20-50	≤ 20	≤ 30	70 to 105
>50	≤ 15	≤ 25	75 to 105

CONCLUSIONS

Tested method for the determination of patulin in raspberry samples is rapid and simple, involving a single extraction step. No enzymatic removal of pectin is required for purees. Obtained results were significantly below the contents of patulin for mold contaminated raspberries reported by other authors, indicating lower patulin production by molds which contaminated raspberries from Serbia in 2010. This method is suitable for the analysis of patulin in frozen raspberries as the finished product and as a feedstock for further processing. Further studies should include monitoring of the patulin presence in fractions of frozen raspberries, which are included in the composition of various food products (fruit yogurt, marmalade, juice, etc.)

ACKNOWLEDGEMENTS

This paper presents the part of research conducted under the project III 46001 Development and utilization of novel and traditional technologies in production of competitive food products with added value for national and global market CREATING WEALTH FROM THE WEALTH OF SERBIA funded by Ministry of science and technological development of Republic of Serbia

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COMPARISON OF METHODS OF DIRECT MICROSCOPY AND DELAVAL CELL COUNTER IN DETERMINING THE SOMATIC CELL COUNT IN MILK SAMPLES

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ABSTRACT: A number of somatic cells in milk indicates the presence of an inflammable process in mammary glands and serves as an indicator of milk hygienic suitability and milk quality. Somatic cells are mostly leukocytes which reflect the condition of the body in the view of physiological and pathological changes and a possible incidence of mastitis. Apart from mastitis, a somatic cell count in milk is also affected by the following factors: physiological-pharmacological (the phase of lactation, race, veterinarian drugs) and stress-related ones (method of feeding, transport, keeping conditions, way of milking, technical validity of milking machines). An elevated somatic cell count has a negative effect on the activity of starter cultures and thermal stability of milk. Determination of the somatic cell count in milk is a key factor in evaluation of milk quality and therefore it is necessary to conduct an adequate milk control for presence of somatic cells. The aim of this work is a statistical evaluation of the results obtained by comparing the reference ISO 13366-1 method for determination of the somatic cell count and the DeLaval equipment.
Key words: *cow milk, somatic cells, direct microscopy, DeLaval counter*

INTRODUCTION

Milk is a white coloured biological liquid of complex composition, and specific taste and smell, which is secreted by the mammary glands of female mammals after calving, and which serves for nutrition of infant mammals. Several hundreds of different substances can be isolated from milk and its basic components include water, proteins, fats, carbohydrates, enzymes and mineral substances. Contents of these components and their proportions in milk are different in different kinds of animals. In cow milk, an average content of water is 87.5%, dry substances 12.5%, milk fat 3.8%, proteins 3.15%.

Apart from chemical substances contained in milk, there are a small number of leukocytes in the healthy udder quarter milk, which originate from blood and serve for defence against infection. Together with the smaller number of epithelial cells (originating from the mammary gland mucosa) the leukocytes constitute somatic cells in milk (Harmon, 1994). Concentration of somatic cells in milk is commonly denoted as a somatic cell count (SCC) and it has long been used as an indicator of the inflammable process in the mammary gland (Katić, 2007). Also, the SCC is an important indicator of the hygiene suitability and quality of milk and thus it is necessary and purposeful to conduct an adequate control for the presence of somatic cells (Hanuš et al., 2011).

An average SCC in the healthy udder milk is 70000-90000 cells per ml and in most cases it is lower than 150000 cells per ml. A somatic cell count in milk and proportions of certain types of leukocytes depend on numerous factors including the season, parity and the phase of lactation, environmental factors and technological procedures applied on the farm, the health status of a mammary gland and secreting activities of the udder cells (Barkema et al., 1998, 1999; Hristov, 2002; Hristov et al., 2006). Also, the intensity of increasing the SCC in milk in individual quarters depends on the type of pathogen. With the somatic cell count ≤ 400.000/ml, the requirements for the hygienic suitability and quality of milk are satisfied.

However, this somatic cell count is not such a good indicator of the prevalence of mastitis in dairy cows. Even with the somatic cell count of 400.000/ml in the bulk milk, clinical and subclinical mastitis may be present in the herd of dairy cows (Katić, 2007).
There are several analytic methods used to determine somatic cells. In addition to the reference method of direct microscopy (ISO 13366-1) and the method which enables fluoro-opto-electronic counting (ISO 13366-2), there are numerous instrumental methods which working principle is based on cell staining and electronic counting. The methods in use are Fossomatic, Chemometec, NucleoCounter SCC–100 and DeLaval counter of somatic cells (DCC). DCC is a portable instrument used for fast and operative monitoring (Gonzalo et al., 2006). A DCC working principle is based on optical and automatic counting of somatic cells. A digital camera takes a picture of somatic cells' nuclei which were previously stained in the cassette with a DNA specific fluorescent reagent. Each cassette has a volume of 60 µl and the measuring range of the DeLaval counter is 10 000–4 000 000 somatic cells/ml (www. DeLaval. com).
The aim of this work is a statistical evaluation of the results obtained by comparing the reference ISO 13366-1 method for determination of the SCC and the DeLaval counter.

MATERIALS AND METHODS

Checking the number of somatic cells in cow milk is a well-established method for timely detection of subclinical mastitis in cows. Because of high frequency of subclinical mastitis and enormous material losses in dairy herds caused by it, it is a common practice to conduct routine scanning of cow milk for the somatic cell counts.
Testing was done on the Holstein-Friesian cow farm (PKB Corporation), which uses a tying system and where milking is performed in split shifts in the cowshed, on the bedding. The experiment was carried on 2.5–3.5-year-aged cows, in the first and second lactation phases, which average milk yield for 305 days of lactation amounted about 7500 liters of milk.
Milk samples were taken immediately before morning milking (20 samples) and after noon milking (20 samples). Forty samples were analysed in total. Samples were checked using a method of direct microscopy BS EN ISO 1366-1 (DMC) and by means of a DeLaval (DCC) counter, in three repeats.

RESULTS AND DISCUSSION

The Duncan's multiple range post hoc test as well as the paired t tests were used in this study in order to find out statistically relevant differences in the somatic cell counts measured by two methods. Test results are shown in Tables 1, 2, 3, 4, 5 and 6. and Charts 1 and 2.
Based on the test results (Table 1), out of 20 samples in total, seven samples (35%) showed concentration ≥ 400 000 cell/ml. The SCC (somatic cell count) ranged from 400 000 to 2 600 300 cell/ml. An elevated somatic cell count indicates poor quality milk, and the samples are hygienically unsuitable. An elevated somatic cell count above 400 000 was determined with the DMC and DCC methods in all three repeats.
Test results of samples after the noon milking (Table 2) indicate a lower presence of somatic cells. Only 2 samples (10%) showed somatic cell concentrations higher than the allowed ones. The SCC ranged from 430 000 (sample 9) to 1054 200 (sample 17). Somatic cells were determined by the DMC and DCC methods. Other samples showed contamination below 400 000 cell/ml.

Table 1. Total somatic cell counts determined by DMC and DCC methods before morning milking (count of cells/ml)

Sample	DMC			DCC		
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3
1.	40 200	42 600	39 300	45 200	47 600	42 600
2.	121 000	126 800	101 400	144 000	137 800	151 000
3.	225 600	220 300	200 300	245 000	250 600	257 300
4.	37 700	39 400	33 700	42 700	44 000	57 100
5.	404 200	410200	400 700	430 200	457 200	400 900
6.	27 300	26 900	27 000	27 100	28 300	26 300
7.	125 300	129 200	136 000	155 400	148 200	123 600
8.	134 300	130 000	126 200	144 200	133 800	134 600
9.	602 200	622 500	598 400	670 300	628 400	647 400
10.	218 300	220 000	215 300	226 400	235 200	215 400
11.	588 600	583 700	580 000	624 300	680 400	593 800
12.	414 200	427 200	431 000	450 300	444 500	431 200
13.	200 600	189 600	192 300	190 600	193 200	182 600
14.	725 300	731 400	722 600	736 300	725 600	743 200
15.	1620400	1618900	1633200	1646 00	1670200	1636 100
16.	41 600	45 200	38 600	35 900	41 300	42 100
17.	2 150 800	2 280 600	2 190 300	2 450 000	2 350 00	2 600 300
18.	52 400	53 000	53 200	56 700	53 400	51 500
19.	22 000	21 400	23 000	24 200	21 000	21 200
20.	32 700	32 400	33 000	31 700	32 400	30 700

DMC - Direct Microscopic Count; DCC - DeLaval Cell Counter

Table 2. Total somatic cell counts determined by DMC and DCC methods after noon milking (count of cells/ml)

Sample	DMC			DCC		
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3
1.	24 200	23 100	19 900	25 200	27 600	22 600
2.	31 000	29 800	31 400	34 000	37 200	32 400
3.	123 200	120 000	122 700	147 200	150 800	139 500
4.	22 500	23 000	23 200	24 600	26 300	24 000
5.	127 300	126 800	127 000	132 100	129 600	131 250
6.	226 400	220 100	237 300	249 200	238 700	244 400
7.	37 300	39 200	36 500	37 400	34 200	42 600
8.	72 400	72 500	78 200	77 300	88 600	72 000
9.	434 300	430 700	426 700	484 200	437 300	512 600
10.	308 300	320 000	317 900	336 200	330 200	356 900
11.	83 200	84 700	80 000	91 200	90 400	93 500
12.	24 600	28 200	21 000	27 800	34 500	31 000
13.	152 600	153 000	150 200	156 900	153 500	151 000
14.	82 000	81 000	83 000	94 600	91 000	91 200
15.	332 000	300 400	318 000	361 700	332 700	330 000
16.	50 200	55 400	48 200	45 400	57 900	52 600
17.	1 047 400	1032 300	1027 600	1029 600	1037 200	1054 200
18.	22 100	23 000	23 600	26 200	23 500	21 500
19.	92 000	91 400	93 000	94 200	111 300	121 600
20.	107 400	112 000	103 400	123 000	106 000	132 400

DMC - Direct Microscopic Count; DCC - DeLaval Cell Counter

The SCC depends on many factors. Vuković (2008) claims that the SCC depends on the time of milking, because there are deviations in the morning or evening milking as well as during the same milking process. Dohoo and Meek (1982) established normal diurnal variations of SCC in milk samples taken at different times during the milking and between the milking processes. Generally speaking, the SCC is the highest at the end of a milking process and the lowest immediately before milking. Also, the SCC is significantly higher in colostrum than in milk. In the first few days after calving, in the first lactation, the SCC was higher than one million per ml of milk, and after two weeks it was about 500000 per ml (Boboš and Vidić, 2005). At the end of lactation, the SCC increases again.

Table 3. Testing the significance of differences in somatic cell counts obtained by DMC and DCC methods regardless the time of milking

Method	Average number of somatic cells	Number of samples (3 repeats)	SD	SE
Direct Microscopic Count	280394,17	120	442021,77	40350,88
DeLaval Cell Counter	299027,92	120	474610,85	43325,84

SD – standard deviation, SE – standard measuring error

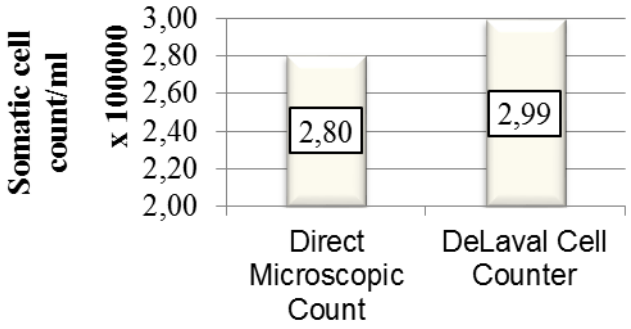

Figure 1. Average somatic cell count by DMC and DCC methods regardless the time of milking (cell/ml)

Table 4. Determination of statistically relevant differences between DMC and DCC methods (t-test) regardless the time of milking (cell/ml)

Pair	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig.
				Lower	Upper			
DMC - DCC	-18633,75	48490,13	4426,52	-27398,71	-9868,79	-4,210	119	0,000

Based on the results of testing (Tables 3 and 4, Chart 1), it was found out that an average somatic cell count per ml of milk, measured by the DMC method, was 280394.17, and when measured by the DCC method it was higher and amounted 299027.92. A paired t-test showed that there was a statistically significant difference in somatic cell counts obtained by using the two methods (t(119)=-4,210, p=0,000), whereby an elevated somatic cell count was determined by DCC method. Hanuš et al. (2010) claimed that the SCC, determined by the DCC, DMC and CMT (California mastitis test), was approximately of the same value and that there were not any statistically significant differences (> 0.92, P < 0.001). Using the DCC and DMC methods for milk analysis, Chaiyotwittayakun et al. (2008) got the similar results, which showed a statistically significant difference. Results of comparing SCCs with regard to the time of milking are given in Tables 5 and 6 and Chart 2 **.**

Table 5. Testing the significance of differences in somatic cell counts obtained by DMC and DCC methods with regard to the time of milking

Milking	Method	Average number of somatic cells	Number of samples	SD	SE
Morning	Direct Microscopic Count	391858,33	60	562073,15	72563,33
	DeLaval Cell Counter	418160,00	60	608821,31	78598,49
Noon	Direct Microscopic Count	168930,00	60	229961,22	29687,87
	DeLaval Cell Counter	179895,83	60	234092,74	30221,24

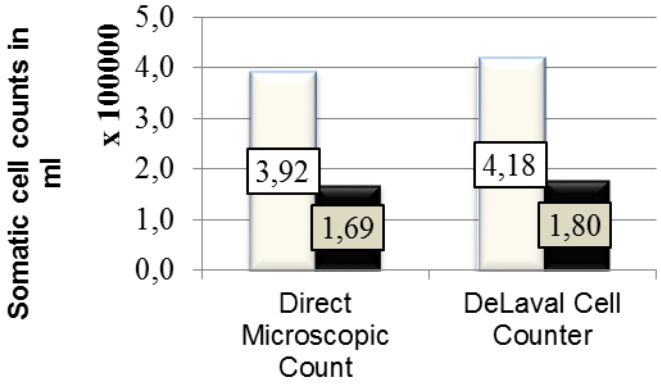

Figure 2. Average somatic cell counts before the morning and after the noon milking (cell/ml)

Table 6. Determination of statistically relevant differences between DMC and DCC methods (t-test) before morning milking and after noon milking (cell/ml)

Pair DMC - DCC	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig.
				Lower	Upper			
morning	-26301,67	66138,16	8538,40	-43386,96	-9216,37	-3,080	59	0,003
noon	-10965,83	15766,92	2035,50	-15038,86	-6892,81	-5,387	59	0,000

A paired t-test showed that there was a statistically significant difference (t(59)=-3,080, p=0,003) in somatic cell counts in milk measured by the two methods in the morning milking since the DCC method showed an elevated somatic cell count, which amounted 418160.00, while the cell count measured by the DMC method amounted 391858.33. Also, for the noon milking, it was determined a statistically significant difference (t(59)=-5.387, p=0.000). Using the DCC method a higher SCC (179895.83) was determined than by the DMC method (168930.00). The SCC is a crucial indicator of milk quality and hygienic suitability. Standardization of methods for determination of the SCC is important for laboratories as well as for veterinarians, manufacturers and milk industry. Statistically significant differences determined between the DCC and DMC methods may result from a human factor (sample preparation, counting errors), calibration procedure on the DeLaval counter, way of sample transfer and sample preservation system, type of milk (cow milk, sheep milk, goat milk, human milk, etc.) (Berry and Broughan, 2007; Hering et al., 2008; Hanuš et al., 2010). Hillerton et al. (2004) analysed the possibilities for an error elimination during the SCC determination by establishing a geometrical mean at the level of 13 values (measuring). Test results definitely support the elimination, but the procedure itself is quite impractical for the fast and operative monitoring.

CONCLUSION

Test results, based on 40 samples of cow milk before the morning and after the noon milking, indicate an elevated somatic cell count in 9 samples (22.5%). The elevated SCC was determined by the method of direct microscopy ISO 13366-1 and by means of the DeLaval counter in all three repeats. Statistical analysis showed that there was a statistically significant difference in somatic cell counts in milk measured by these two methods. Namely, the SCC detected was higher when using the DCC method than when using a reference method of direct microscopy. A method of direct microscopy enables an accurate detection of SCC but only if the protocols concerning transfer, preservation, preparation and analyst's skills are fully observed during the sample analysis. A DeLaval counter is portable, requires a small-sized sample, and makes an excellent alternative to expensive counters when fast and operative monitoring is required.

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DETERMINATION OF LIPOSOLUBLE VITAMINS CONTENT IN DIETETIC PRODUCTS

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ABSTRACT: A large number of dietary products contains high content of vitamins A, E and D. Depending on usage, the amount of consumed vitamins can significantly surpass the recommended daily intake. Also, if the content does not match the declared, this can lead to consumers misleading. In our market, there is a large offer of dietary products in pharmaceutical forms, as well as enriched foods with these vitamins content declared. By proper use of these foods, it is possible to largely meet the daily requirements for vitaminsOur goal was to determine liposoluble vitamin content in products where their content is declared by using HPLC and UPLC methods with spectral and fluorescent detector. For successful determination of the content of vitamins A, E and D in the complex composition of foods multiple purification of samples is necessary, while for the determination of the synthetic products it is enough to apply the extraction. We applied the extraction with n-hexane, extract steaming and reconstitution in methanol, with membrane filtration. Separation was performed with RP-HPLC method with fluorescent detector with variable wavelengths, and UPLC method with the spectral detector. The dietary products of different backgrounds, forms and methods of production (total 148) were analyzed, of which 8.3% had lower, and 2.1% higher content than declared.
Key words: *vitamin A, vitamin E, vitamin D, HPLC, UPLC, dietary products*

INTRODUCTION

Dietary supplements are foods that supplement the normal diet and which are concentrated sources of nutrients (vitamins, minerals and other substances with a nutritional or physiological effect). They are marketed in dose forms (capsules, tablets, drops, etc.). The oldest and most complete regulation on dietary supplements is a U.S. regulation. The basic law regulating the manufacture, sale, labelling and marketing of dietary supplements is the Dietary Supplement Health and Education Act (DSHEA) from 1994.

The EU regulation on food supplements is only partially harmonized. Directive 2002/46/EC on the approximation of legislation of the Member States relating to food supplements is determined list of permitted vitamins and minerals, substances that are used as their sources, marking units and permitted forms of dietary supplements. Maximum allowable amounts for vitamins and minerals, herbal supplements and health statements are not yet harmonized and apply national regulations that differ as a barrier to trade dietary supplements in the EU. (Orientation paper on the setting of maximum and minimum amounts for vitamins and minerals in foodstuffs, 2007). In Serbia, there is a regulative about dietary supplements.

Required amounts for people of different age and lifestyle are constantly adjusted, as well as the opinion about the preffered way of intake: by food, or by precisely dosed dietary supplements. It is certainly rational to decide is it justified to add supplements to everyday diet before actually applying them. (Ball GFM, 2004).

The most common method for analyzing vitamins A, E and D and its analogs in pharmaceutical preparations, feedstuffs, and tissues is ultra-performance liquid chromatography combined with a UV or high-performance liquid chromatography combined with fluorescence detectors.

A large number of dietary products have a high content of vitamins and minerals. Depending on usage, the amount of nutrients entered can significantly exceed recommended daily

intake. Also, if the content does not match the declared quantity, consumers can be misled. In our market, there is a more diverse supply of dietary products in pharmaceutical forms, as well as foods fortified with declared content of vitamins and minerals. by the proper use of these foods, it is possible to substantially meet the daily needs. Dietary supplements sometimes change contents, under the same generally accepted name.
In food, vitamines A, D and E are found along with other substances, that interfere in process of determination vitamin contents. These substances can be removed in different ways. Saponification removes glycerides, peroxydes and partially pigments, but cannot remove steroles and carotenoides. For dividing tocopheroles from other substances, as well as for dividing different tocopheroles, different methods can be used, but liguid and gas chromatography are the most common (Delgado-Zamerreno MM et al. 2001; Sanchez-Perez et al. 2000; Blake CJ 2007).
Our goal was to determine liposoluble vitamin content in products where their content is declared by using HPLC and UPLC methods with spectral and fluorescent detector.

MATERIAL AND METHODS

In analysis, analytical vitamin standards (manufacturer: Sigma Co, St Louis, MO, USA) were used to prepare solutions for determination of detection limits and quantification limits (series of solutions from 0,01 to 0,5 μg/mL), and solutions for calibration curve (1,0, 2,5, 5,0, 10,0 and 20,0 μg/mL). Methanol and acetonitrile were used (HPLC purity, manufectured by Merck, Germany), hexane (HPLC purity, manufectured by Merck, Germany), water of HPLC purity (water demineralized and purified using commercial „Millipore Milli-Q“ system). Other chemicals were of analysis purity (p.a.).
In our laboratory, HPLC method with fluorescence detecor was used for determination of vitamin A and E. UPLC method with PDA detector was used for determination of vitamin D_3. Operational conditions were given in table (Table 1).

Table 1. Operational conditions

HPLC conditions:	
Pump:	Waters M600 E,
Injector:	Rheodyne 7125, loop 20μl
Column:	RP-HPLC C8, 5 μm,
Detector:	RF-535 Shimadzu, Fluorescence HPLC
Mobile phase:	95% CH_3OH isocratic elution
Flow rate	1,0 mL/min
Column temp	~20 ^{0}C
Wavelengths:	λ_{ex} = 295 nm, λ_{em} = 330 nm for vitamin E λ_{ex} = 325 nm, λ_{em} = 475 nm for vitamin A
Acquisition:	Clarity chromatography station for Windows

UPLC conditions	
LC system:	Waters ACQUITY UPLC System with PDA detector
Mobile phase:	Solvent A: water: acetonitrile (90:10) Solvent B: acetonitrile : methanol (50:50)
Injection volume:	5 μl
Flow rate:	0,7 mL/min
Column temp:	35 ºC
Column:	ACQUITY BEH C18, 2,1 mm x 100 mm 1,7 μm
UVmax: 265 nm	

RESULTS AND DISCUSSION

Using modern instrumentation technique (HPLC, UPLC) with different detection systems, 148 dietetic products were analyzed during the past year. Presence of low vitamin D3 content could be determined using UPLC-PDA method (Figure 1) while for determination of vitamin A and E content HPLC with fluorescent detector was chosen which is enough specific and sensitive for their determination (Figure 2 and Figure 3). Applied methods were validated. Quantification limits were determined: 0,1 µg/mL for vitamins A and E and 0,01 µg/mL for vitamin D_3. Linearity for solutions from 0,1 to 20 µg/mL and from 0,01 to 10 µg/mL was determined, as well as the surface of appropriate peaks. Calibration curves were constructed as a result of four injections for each concentration level. Correlation coeficients are: r=0,9995 for vitamin A, r=0,9992 for vitamin E and r=0,9991 for vitamin D_3. Accuracy of the method was examined by determining recovery for standard solutions of 10 µg/mL (RSD = 98,2% for vitamin A, 97,5% for vitamin E and 97,3% for vitamin D_3). The dietary products of different backgrounds, forms and methods of production (total 148) were analyzed, of which 8.3% had lower, and 2.1% higher content than declared (Table 2). All differences were less than 50% of declared values, which confirms the problem lies in bad technological process or bad material, and not an incident.

Table 2. Accordance of dietetic products to the declared values of vitamins A, E and D

examined parameters	examined samples		
	correct	not correct	
		higher	lower
Vitamin E	127	1	2
Vitamin A	55	2	7
Vitamin D	24	--	3
Total samples*	148	15	

** Some content several vitamins*

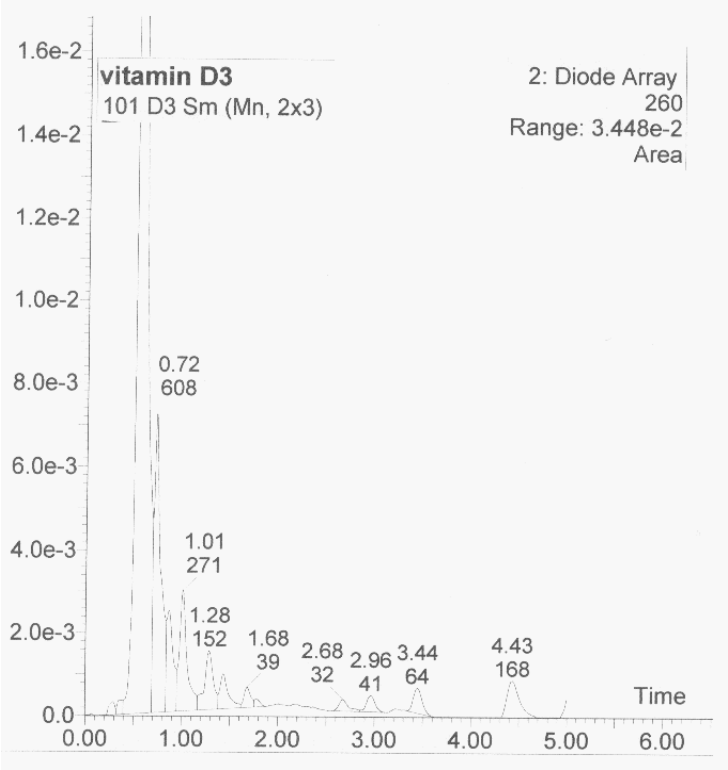

Figure 1. Vitamin D in infant formula sample

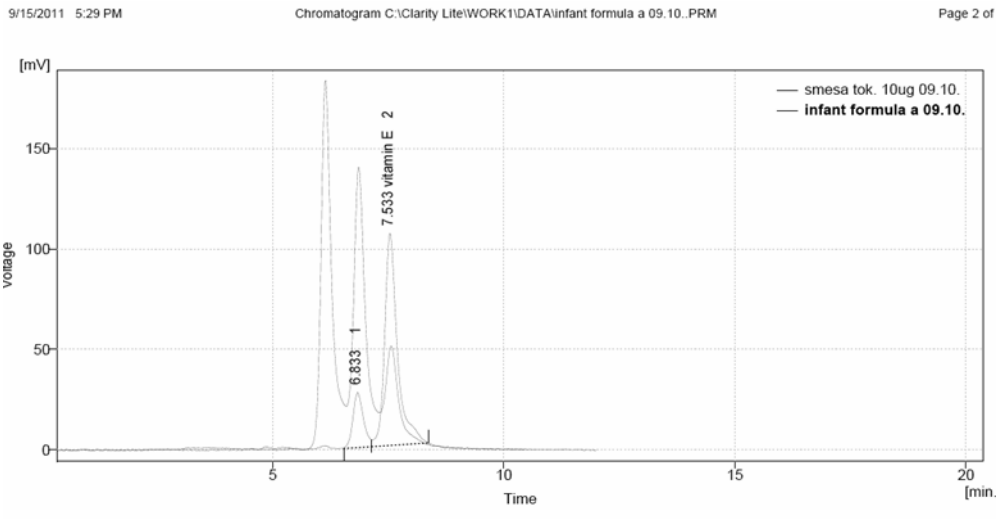

Figure 2. Vitamin E content in infant formula with natural tocopherols

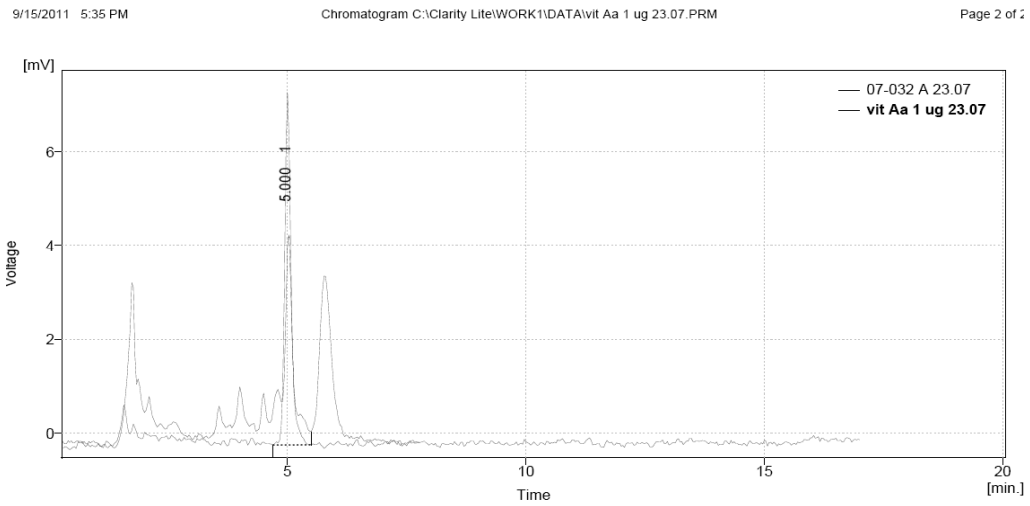

Figure 3. Vitamin A in infant formula

CONCLUSIONS

Adoption of regulations on food supplements based on the new Law on Food and harmonized with EU regulations and international standards is the basis for introduction of making order in the production and distribution of this specific group of foods and for health protection and other consumers' interests. Analyzed samples of dietary supplements mainly respected declared limitations for vitamins A, E and D_3. Those that didn't cannot endanger health, if used as proposed, but they significantly change total daily intake of these vitamins.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science and Developmental of the Republic of Serbia (Grants no. 31069)

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INFLUENCE OF DIFFERENT STORAGE TEMPERATURES ON SAFETY OF DIARY PRODUCTS

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ABSTRACT: The experiment was designed to monitor durability of certain diary products stored at proper temperatures (8^{o}C) and elevated temperatures (14^{o}C) within their shelf-life. Samples of fermented milk products were tested during 25 days, samples of cheese spread products over 80 days, while soft white cheese samples were analyzed during a storage period of 100 days. In the defined study periods, depending on the type of product, pH and a_w value of the product, as well as sensory analysis (odor, taste, color and consistency), and microbiological safety were investigated. The above investigations were performed in accordance with National legislation. Research results indicate that products stored at 14°C showed significant acidity (lower pH value), changed sensory properties, as well as an increased number of aerobic bacteria.
Key words: *milk products, shelf life, storage temperature, quality.*

INTRODUCTION

It is well known that, depending on numerous internal and external factors, microorganism growth occurs during diary products storage resulting in sensory changes, i.e. spoilage. This is due to the fact that if storage conditions are inadequate, nutrients in milk products are a good medium for growth and development of individual groups of microorganisms.
The diverse range of dairy products found on the market today is a result of knowledge and experience acquired over the years. However, the contemporary consumer sets high requirements for the producer, expecting him to stay abreast of modern trends, related to both high quality and product safety.
In other words, the consumer's primary requirement is that during the estimated time of storage the food is safe, as well as not subject to adverse changes (Gregory et al, 2006). The length of shelf life of milk products is primarily influenced by: the number and type of microorganisms present in raw milk as the primary raw material, state of packaging material, hygienic and sanitary conditions during the production cycle (washing and disinfection of equipment, pasteurization temperature regimes), as well as by the storage temperature of the final product.
In most cases, microorganisms that cause the spoiling of milk and dairy products (Mostert and Jooste, 2002; Delacroix-Buchnet, 2004), to some extent reduce their quality which results in significant economic loss (Randolph, 2006). Reduced quality is a result of different chemical and biochemical bonds which cause the change in the appearance, odour, texture, taste and aroma of products (Robertson, 2009). The appearance of those bonds is conditioned by metabolic degradation of some components of the product either by microorganisms causing the spoilage or by their enzymes (Randolph, 2006).
Yogurt is known as a good nutritive medium, with a relatively low pH, favorable for the development of acidophilic microorganisms, as opposed to cheese which is less acid and contains less water, as well as added salts, resulting in low a_w value, thus partially limiting growth and multiplication of certain microorganisms. In addition, with its firm consistency, cheese in a manner limits mobility of the microorganisms that cause spoilage (ICMSF, 2005).

MATERIAL AND METHODS

The experiment was designed to monitor durability of certain diary products (fermented milk products, cheese spreads, and soft white cheese), that were stored at proper temperatures (8^{o}C) and elevated temperatures (14^{o}C), within their shelf-life.
Samples were taken from each group of products, in original packaging units, immediately after production, and kept at predefined temperatures (8 and 14^{o}C)
Microbiological, physicochemical and sensory investigations were performed according to a plan, which included testing of the finished product immediately after manufacture, testing in the middle of the declared shelf-life, and at the end of shelf life.
Microbiological examination of diary products was performed in accordance with the Regulations of General and Special Conditions of Food Hygiene at any Stage of Production, Processing and Trade ("Official Gazette RoS", No. 72/2010), as well as with the elements of self-control prescribed in HACCP plans.
Concretely, 25 g of each sample were transferred to a sterile stomacher bag and 225 mL of saline-peptone water was added and mixed for 30 seconds in the stomacher. Further decimal dilutions with the same diluents were made, and the following analysis were carried out on duplicate agar plates: (a) total viable count on Peptone Caunt Agar - ISO 4833:2003; (b) lactic acid bacteria (LAB) on MRS agar incubated with a double layer for 48 h at 30^{o}C; (c) *Escherichia coli* on VRB agar – ISO 16649-1, 2; (f) *Staphylococcus aureus* on Baird Parker medium with added egg yolk tellurite emulsion - ISO 6888-1:1999; (g) yeast and moulds on Sabouraud-4% Maltose agar – ISO 21527-1, 2. Determination of the presence of *Listeria monocytogenes* was carried out following the procedure of ISO 11290−1, 2:2004, and *Salmonella spp.* – ISO 6579:2002.
The physicochemical investigation performed determined pH values (pH meter, MA-5730, No 35 398 PAT, Iskra) and water activity (a_w), using a hygrometer (Wert-Messer, Durotherm), at a constant temperature of 25ºC.
Sensory analysis of products encompassed odor, taste, color and consistency.

RESULTS AND DISCUSSION

Results of physicochemical investigation of milk products during storage are presented in Table 1.
Change of pH in fermented milk products stored at 8^{o}C was statistically significant $p<0.05$ (Day 1, pH=4, 425±0.014, at the end of storage pH=4, 26±0.093). However, for products stored at 14^{o}C, midway through their storage life, pH reduction (3.96±0.040) was established with statistically significant $p<0.01$, at the end of the cycle reaching 4.01±0.017. These products had altered sensory properties with a tendency toward higher acidity.
Change of pH-value in samples of cheese spreads, in both groups was statistically highly significant, $p <0.01$, where was not statistically significant differences in the samples stored in a 14^{o}C, from the 40 to 80 days. At the end of shelf life, these products also had altered sensory properties (rancid taste).
The established pH-values, at soft white cheeses stored at 8 ° C, from 1 to 50 days was not statistically significant ($p < 0.05$), but at the end of of the cycle reaching (100.day) was statistically significant, $p <0.01$ (Day 1, pH=4.82, Day 100, pH=4.65), as opposed to cheese samples stored at 14°C, where, already in mid shelf life, there was a reduction of pH, (statistically significant $p<0,001$) accompanied by a change of sensory properties (bitterness, altered structure, unpleasant odor).

Table 1. Results of the physicochemical testing of products (a_w and pH-value) during storage

Type of product	**Storage temperature**	**Days of testing**		
Fermented milk products		Day 1	Day 13	Day 25
		pH value (Ā±SD)		
	8°C	4.425±0.014	4.20±0.013	4.26±0.093
	14°C	4.43±0.035	3.96±0.040	4.01±0.017
Cheese spreads		**Days of testing**		
		Day 1	Day 40	Day 80
		pH value (Ā±SD)		
	8°C	4.68±0.023	4.61±0.010	4.52±0.014
	14°C	4.71±0.021	4.65±0.013	4.66±0.007
		a_w value		
	8°C	0.962	0.968	0.966
	14°C	0.964	0.966	0.973
Soft white cheese, 45% milk fat in dry matter		**Days of testing**		
		Day 1	Day 50	Day 100
		pH value (Ā±SD)		
	8°C	4.82±0.015	4.73±0.014	4.65±0.018
	14°C	4.83±0.012	4.15±0.010	3.64±0.007
		a_w value		
	8°C	0.958	0.966	0.968
	14°C	0.959	0.977	-

Legend: Ā – average value; SD − standard deviation

For cheese and cheese spreads stored at 8°C, during the investigation period the a_w value was uniform (approximately 0.96), while for samples stored at a higher temperature, this value was somewhat elevated (0.977) in mid shelf life. At the end of shelf life, the a_w value was not measured because samples shower pronounced signs of spoilage.

Table 2. Results of microbiological testing of products during storage

Type of product	**Storage temperature**	**Days of testing**		
Fermented milk products		Day 1	Day 13	Day 25
	Total number of LAB, CFU/ml			
	8°C	$3.4x10^8$	$1.7x10^8$	$2.8x10^7$
	14°C	$3.4x10^8$	$1.4x10^8$	$2.3x10^7$
Cheese spreads		**Days of testing**		
		Day 1	Day 40	Day 80
	Total number of aerobic bacteria, CFU/g			
	8°C	θ	θ	Θ
	14°C	θ	60	200
Soft white cheese, 45% milk fat in dry matter		**Days of testing**		
		Day 1	Day 50	Day 100
	Total number of aerobic bacteria, CFU/g			
	8°C	θ	θ	θ
	14°C	θ	150	6000

Legend: CFU – colony forming units; LAB − lactic acid bacteria; θ - not detected

Microbiology test results showed that tested samples of dairy products did not contain *L. monocytogenes*, *E. coli*, *Salmonella* spp, *Staphylococcus aureus*, or yeasts and molds. Finished products, as well as products during the storage period were safe for use.

The lactic acid bacteria count in fermented milk products was characteristic for this type of product, but also consistent during the storage period, at both investigated temperatures. During the storage period, at a storage temperature of 8°C, no aerobic bacteria were found in samples of cheese spreads and soft white cheese, while at 14°C their number grew until Day 40 i.e. Day 50 of storage.
Investigation results of Huskar et al., (2003), showed that during the storage process of probiotic yogurt at temperatures of 4 and 20^{o}C there is a change of quality of stored products, and that changes are more pronounced at higher storage temperatures. The decrease in product quality is linked to increased content of lactic and citric acid.
For investigations of microbiological processes in butter during the storage period and cooling temperatures, Simonovic (1986) indicates that the increase of the total microorganism and lipolytic bacteria count is intensive after Day 15, and that on Day 30 at 4^{o}C this count was increased by up to 10 times. The same author states that the change in the lipolytic bacteria count is directly proportionate to the increase in the total microorganism count.
By testing the effect of various storage temperatures (-12, 2, 8 and 12^{o}C), as well as the presence of salt on microbiological changes in five different types of thermo processed products, Jensen et al., (1983), state that lower storage temperatures and the presence of salt have a significant effect on reducing the total number of microorganisms, *Coli* bacteria and yeasts, compared to their counts established in products stored at higher temperatures.
The effect of various storage temperatures is directly linked to lipolytic and proteolytic processes taking place in the product itself, with an intensity directly dependent on external temperature. This is corroborated by results of Sheehan et al., (2004), stating that the intensity of proteolyses is more pronounced in samples of Mozzarella cheese stored at higher temperatures (the experiment was done at 4 and 12°C).
Demarigny et al., (2006), showed that “mini cheeses” stored at 18^{o}C for 12 weeks have altered sensory properties compared to cheeses stored at 7^{o}C for 24 weeks. UHT milk samples, taken in the spring and fall from five different dairies distributed in different regions of France (north, north-west, south-west and central France), were stored at various temperatures (4, 20 and 40^{o}C), and analyzed after 21, 42, 62, 90, 110 and 180 days, and it was established that higher temperatures (40^{o}C) have a pronounced effect on pH decrease and the increase in non-casein (Gaucher et al., (2008). In other words, the sustainability of dairy products, as well as their shelf life directly depend on storage temperature.

CONCLUSION

Results of this research enable following conclusions:

- Investigated dairy products stored at 8^{o}C showed no significant pH decrease, as opposed to products stored at higher temperatures (14^{o}C). On the other hand, established a_w values for cheese spreads and UF cheeses showed no variation during shelf life.
- Test results for sensory properties of fermented milk products stored at 14^{o}C demonstrated a tendency toward acidity increase during the investigation period, and for cheese spread samples a rancid taste was also established.
- For cheese samples stored at 14^{o}C, in mid storage period (Day 50), a bitter taste, altered structure, as well as a certain unpleasant odor, were established, with a significantly reduced pH.
- Results of microbiological testing showed that during shelf life investigated products were microbiologically acceptible. No presence of pathogenic microorganisms was established. On the other hand, elevated storage temperatures influence an increase of the total bacteria count, mainly accompanying a change of sensory properties, as well as a decrease of pH value.

ACKNOWLEDGEMENTS

The results presented in this paper are part of Project III, No 46009: "Improvement and development of hygienic and technological procedures in production of foodstuffs of animal origin with the aim of producing high-quality and safe products competitive on the global market" funded by the Ministry of Education and Science of Serbia.

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INFLUENCE OF THE HERB EXTRACT ON INHIBITION OF BEEF MEAT SPOILAGE - POTENTIAL SOURCE OF NATURAL PRESERVATIVE

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ABSTRACT: Raw beef can be contaminated by microorganisms and support the growth of pathogens, and may lead to serious food-borne diseases. In many cases, plant extracts exibit antimicrobial and antioxidant activity. We investigated the inhibitory activity of 2.5% ethanol extract of Serbian herb *Kitaibelia vitifolia* against ATCC strains: *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus hauseri*, *Proteus mirabilis*, *Bacillus subtilis* and fungi *Candida albicans* and *Aspergillus niger*, in a lean beef meat. We prepared samples (48 pieces per 25 g), according the sterility demands, from one piece of beef (2 kg). We organized 3 experimental group: samples from first (I - control) group are non-treated; II - samples immersed in sterile water and III - samples immersed in above mentioned herb extract. Analysis was carried out during storage at 4 ^{0}C and 25 ^{0}C (0. d, after 2, 4 and 7 d). Longest sustainability shows samples from experimental group III, on both temperature of storage, determined by method for proving spoilage (Nessler). Antimicrobial activity evaluated by their minimum inhibitory concentrations (MIC). The above mentioned extract showed strong inhibitory activity against *E.coli* (7.820 mg/mL), *S. aureus*, *P. mirabilis and K. pneumoniae* (15.625 mg/mL). Moderate sensibility on applyed herb extract shown *P. hauseri* (31.250 mg/mL) and minimum of inhibitory activity against *B. subtilis* (62.500 mg/mL). Among fungi, *A.niger* is very susceptible (7.820 mg/mL), unlike the *C. albicans* (62.500 mg/mL). This extract may be further investigated as a natural preservative to the food industry.
Key words: *beef, spoilage, antimicrobial activity, herb extract, Kitaibelia vitifolia*

INTRODUCTION

Diseases caused by eating food contaminated with pathogens around the world have strong economic, as well as the impact on public health (Gandhi and Chikindas, 2007). Microorganisms can adapt to survive and grow in a wide level of ambient conditions and the variety of raw and processed foods, including milk and dairy products, various kinds of meat and meat products and fresh products. Failure of food includes physical and chemical changes, oxidation, discoloration, or the occurrence of unpleasant taste and odor, as a result of the growth of microorganisms and their metabolic products (Gram et al., 2002). Spoilage of chilled meat is mainly caused by *Pseudomonas species*, which cause taste and odor, discoloration, production of gas and mucus (Oussalah et al., 2006a). Many pathogenic microorganisms including *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni, Candida spp., Zygosaccharomyces spp., Fusarium spp., Aspergillus spp., Rhizopus spp., Penicillium spp.,* and *Salmonella spp.* have been identified as causes of "food-borne" disease or spoilage of foods (Betts et al., 1999; Solomakos et al., 2008).
Davidson et al. (2000) reported that the natural herb extracts are known to have huge antimicrobial activities and listed as Generally Recognized As Safe (GRAS). Many natural herbal extracts contain mainly phenolic compounds, which are potent antioxidants (Wong et al., 1995), and occur only in materials of plant origin. Resistance to antibiotics has become a subject of global concern and interest. In recent years there is a growing number of cases of multiple resistance in terms of pathogenic microorganisms in the human population, mainly

due to reckless commercial use of antibacterial drugs widely used in the treatment of infectious diseases (Westh et al., 2004).
This fact reinforces scientists that are exploring new antimicrobial substances originating from various sources, such as medicinal plants. This investigation is continuing by screening a large number of plant families (Parekh and Sumitra, 2007). Ghasemi et al. (2010) determined the antimicrobial activity of the extracts of eight plant species which are endemic in Iran, by agar disc diffusion and serial dilution assays. Most of the extracts showed a relatively high antimicrobial activity against all the tested bacteria and fungi. Some studies show that plant extracts useful for reducing pathogen contamination in meat (Mytle et al., 2006; Ahn et al. 2007), while others report that the very low or no antimicrobial activity against *L. monocytogenes* or *Salmonella* when they are essential plant oils applied to the beef or chicken (Uhart et al., 2006; Firouzi et al., 2007).The aim of our study is to determine antimicrobial influence of ethanol extract of Serbian herb *Kitaibellia vitifolia* on delaying of the lean beef meat spoilage, at usual temperatures of storage (4 ^{0}C and 25 ^{0}C).

MATERIAL AND METHODS

Plant material

Kitaibelia vitifolia is an imposing & undemanding Mallow from ex-Yugoslavia. Above-ground part of the test plant was collected in Central Serbia, in May 2009, at the flowering stage. Taxonomy of plant was identified and the voucher specimen was deposited at the Department of Botany, Faculty of Biology, University of Belgrade (16350 BEOU, Lakušić Dmitar).

Preparation of extract

Herb samples (10.0 g) were extracted by 96 % ethanol as a solvent. The extraction process was carried out using an ultrasonic bath (Brason and Smith-Kline Company, B-220) at room temperature for 1 hour. After filtration, 5 mL of the liquid extract was used for extraction yield determination. The solvent was removed by a rotary evaporator (Devarot, Elektromedicina, Ljubljana) under vacuum, and was dried at 60 ^{0}C to constant weight. The dried extracts were stored in glass bottles at 4 ^{0}C to prevent oxidative damage until analysis.

Spectrophotometric measurements

Spectrophotometric measurements were performed using a UV-VIS spectrophotometer MA9523-SPEKOL 211 (ISKRA, Horjul, Slovenia).

Samples

Samples of lean beef meat (48 pieces per 25 g) prepared from one piece of beef (2 kg). To simulate the usual way of meat preparing, samples split up and macerated using a knife (scalpel), according the sterility demands, in laminar flow biological safety cabinet Iskra IBK 1H2. Samples were measured on a technical scale (KERN EW 150-3M, Kern/Sohn GmbH, D-72 336 Balingen, Germany, M-2-60, CE 07, ISO 9001). We organized 2 experimental group (EG): twenty four samples from control (C) group are immersed in sterile buffered peptone water (8.5 g NaCl, M = 58.44 g/mol, JUS H. G2. 081, 9431, pro analysi, „ZORKA Pharma“ a.d. Šabac and 1 g of Pepton - Pepton-1, Lot 404001, Institute for virology, vaccines and serums, Vojvode Stepe 458, 11221 Belgrade, Serbia) in 1000 mL distilled water) and prepared solution sterilised by autoclaving, at 121 ^{0}C during 20 minutes; EG I - twenty four samples immersed in above mentioned 2.5 % herb extract (pH = 4.60). Duration of immersing the samples in the study groups was 5 minutes. Excess solution from samples of both (C and EG I) group was removed by placing the dipped pieces onto a sterilized paper towel before transferring them to sterile flask for incubation. Qualitative physic-chemical analysis was carried out during storage at 4 ^{0}C and 25 ^{0}C (0 d to 7 d) and micro dilution method performed with watery meat extract on the 7 d of the experiment.

Antimicrobial activity

Minimum inhibitory concentrations (MIC) of the sausage extract and against the test bacteria were determined using a micro dilution method in 96 multi-well micro-titer plates (Satyajit et al., 2007). All tests were performed in Muller-Hinton broth (MHB) with the exception of yeast, in which case Sabouraud dextrose broth was used. A total of 100 µL stock solution of sausage extract (in ethanol, 200 µL/mL) was pipetted into the first row of the plate. Fifty µL of Mueller-Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a final concentration of 0.5% (v/v) for analysis of sausage extract) was added to the other wells. Fifty µL from the first test wells was pipetted into the second well of each microtiter line, and then 50 µL of scalar dilution was transferred from the second to the twelfth well. Ten µL of resazurin indicator solution (prepared by dissolution of a 270-mg tablet in 40 mL of sterile distilled water) and 30 µL of nutrient broth were added to each well. Finally, 10 µL of bacterial suspension (10^6 CFU/mL) and yeast spore suspension (3×10^4 CFU/mL) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked. The standard antibiotic Amracin was used to control the sensitivity of the tested bacteria, whereas Ketoconazole was used as the control against the tested yeast. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37 ^{0}C for 24^h for the bacteria and at 28 ^{0}C for 48^h for the yeast. Color change was then assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value.

Test microorganisms

The antimicrobial activity of the herb extract was tested against the following bacteria: *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153, *Bacillus subtilis* ATCC 6633, and fungi: *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404, in meat extracts prepared from examined lean beef meat pieces, after 8 d of storage. The fungi were cultured on potato-glucose agar for 7 d at room temperature of 20 ^{0}C under alternating light/dark conditions. Then they were cultured on a new potato-glucose substrate for another 7 d. The culturing procedure was performed four times, after which pure cultures required for determination were obtained. The identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Microbiology, Institute Torlak, Belgrade, Serbia.

Determination of microbial spoilage

To assess the freshness and quality of examined meat sample during various ways of storage was determined the bound ammonia (easily hydrolyzable nitrogen) with *Nessler* reagent (tetraiodomercurate bipotasic solution in potassium hydroxide), which prepared with 5 g potassium iodide dissolved in 5 cm^3 of hot water in an Erlenmeyer flask. Hot saturated solution of mercuric chloride added until the precipitate formed is no longer dissolved. After cooling the solution separate, decant a 100 cm^3 volumetric flask. Add 15 g potassium hydroxide dissolved in 30 cm^3 water and bring to volume with water. Add 0.5 cm^3 saturated solution of mercuric chloride, allow to make the solution above the precipitate and separated by decantation, pass in a clean dark bottle and kept from light.

The reaction of the meat extract with Nessler's reagent may lead to the formation of turbidity and change of color and indicate slight decomposition. If a precipitate forms, the decomposition is advanced.

RESULTS AND DISCUSSION

The results on antimicrobial effects of herb extract obtained by the micro dilution method are shown in Table 1. As it can be seen from the results, MICs were determined for 8 indicator strains, and revealed that ethanol herb extract of *K. vitifolia* showed inhibitory effects tithing the concentration range of 7.820 µg/mL to 62.500 µg/mL.

Above mentioned herb extract was exhibited strongest inhibitory activity against *E.coli* (7.820 µg/mL), *S. aureus*, *P. mirabilis and K. pneumoniae* (15.625 µg/mL). Moderate sensibility on applyed herb extract shown *P. vulgaris* (31.250 µg/mL). Lowest inhibitory activity herb extract exhibited against *B. subtilis* (62.500 µg/mL). Among fungi, *A. niger* is very susceptible (7.820 µg/mL), unlike the *C. albicans* (62.500 µg/mL). These results are very similar like results were reported by Kurcubic et al. (2011), obtained in examination of the same influence in fermented dry Sremska sausages, but different of data presented by Mašković et al. (2011) for determination of MIC of the ethanol extract of *K. vitifolia* and standard drugs for same 8 indicator strains, *in vitro*.

Our opinion is that the reasons for this differences arising form interaction between microbes and constituents of meat or some other microorganisms from meat, which alter their ability for defense responses, or protect some examined strains. Generally, the efficiency of most antimicrobial properties of natural supplements can be reduced by the action of certain food components (Glass and Johnson, 2004). Cutter (2000) suggested that the use of herb extracts may afford some reductions of pathogens on beef surfaces, because the antimicrobial activity may be diminished in ground beef by adipose components.

Table 1. Minimum inhibitory concentrations (MIC) of the ethanolic extract of *K. vitifolia* for microbial strains in examined samples of meat extract

	MIC (µg/mL)			
Microbial strains	***ATCC number***	***Meat extract of EG I***	***Amracin***	***Ketoconazole***
		Bacteria		
Staphylococcus aureus	*25923*	15.625	0.970	/
Klebsiella pneumoniae	*13883*	15.625	0.490	/
Escherichia coli	*25922*	7.820	0.970	/
Proteus vulgaris	*13315*	31.250	0.490	/
Proteus mirabilis	*14153*	15.625	0.490	/
Bacillus subtilis	*6633*	62.500	0.240	/
		Funghi		
Candida albicans	*10231*	62.500	/	1.950
Aspergillus niger	*16404*	7.820	/	0.970

Table 2. The dynamic of investigated qualitative physic-chemical changes in stored macerated lean beef meat pieces

Samples (12 per group)	**Nesller reaction**							
	day of storage							
	0 d	**1 d**	**2 d**	**3 d**	**4 d**	**5 d**	**6 d**	**7 d**
C (4 ^{0}C) C_1 to C_{12}	**N**	**N**	$C_{9,10}$: **P** $C_{4,5,6,7,8,11}$: **PP** C_{12}: **U** $C_{1,2,3}$: **N**	$C_{9,10}$: **P** $C_{4,5,6,7,8,11}$: **PP** C_{12}: **U** $C_{1,2,3}$: **N**	$C_{8,10,11,12}$: **P** $C_{1,2,3,4,5,6,7,9}$: **N**	C_1 to C_{12}: **P**	-	-
C (25 ^{0}C) C_{13} to C_{24}	**N**	**PP:**4 **U:** 8	C_{13} to C_{24}: **PP**	C_{13} to C_{24}: **P**	-	-	-	-
EG I (4 ^{0}C) EG_1 to EG_{12}	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**
EG I (25 ^{0}C) EG_{13} to EG_{24}	**N**	**N**	$E_{23,24}$: **PP** $E_{19,20}$: **U** $E_{13,14,15,16,17,18,21,22}$: **N**	$E_{23,24}$: **PP** $E_{19,20,21}$: **U** $E_{13.14.15.16.17.18,22}$: **N**	$E_{22,23,24}$: **PP** $E_{19,20,21}$: **U** E_{13} to E_{18}: **N**	$E_{22,23,24}$: **P** $E_{19,20,21}$:**PP** E_{13} to E_{18}: **U**	E_{13} to E_{24}: **P**	-

*The qualitative results are presented by four categories of reaction interpretation: Positive (**P**); Poorly Positive (**PP**); Uncertain (**U**), and Negative (**N**).*

As shown in Table 2, only the samples from the EG I stored at 4 ^{0}C remained fresh in the absence of bound ammonia during 7 days of storage. In other experimental groups studied discoloration of meat extract were observed on the first day of storage (4 samples in the control group kept at 25 ^{0}C poorly positive and 8 were expressed uncertain reaction, followed by unspecified unpleasant odor). All 12 samples from the control group, which were stocked

at 4 ^{0}C were strongly altered (P) on the fifth day of storage (followed with discoloration), and when stored at 25 ^{0}C for three days. Changes of the color when performing Nessler reaction in samples EG I kept at 25 ^{0}C began the third day, and on the fifth day only 3 samples (E_{22}, E_{23} and E_{24}) were positive, and sixth day all samples above mention experimental group were positive. Qualitative physic-chemical changes confirmed the strong protective antimicrobial activity of the tested plant extracts.

CONCLUSIONS

The present study confirmed the antimicrobial efficiency of the ethanol extract of the Serbian herb *Kitaibelia vitifolia*, and benefits for the sustainability and safety of beef meat (improving microbiological quality and prolonging the shelf-life of the beef to day seven of examination). Above mentioned extract showed relatively high antimicrobial activity against all the tested bacteria and fungi, and may protect consumers from the hazards of food-borne illness. The present study suggests that the extract of *K. vitifolia* is a potential source for the food industry as a natural antibacterial agent.After this screening, further work should be performed to describe the antimicrobial activities in more detail as well as its activity *in vivo*.

ACKNOWLEDGEMENTS

The work is part of the research project “Unapređenje i razvoj higijenskih i tehnoloških postupaka u proizvodnji namirnica životinjskog porekla u cilju dobijanja kvalitetnih i bezbednih proizvoda konkurentnih na svetskom tržištu” (“Improvement and development of hygienic and technological procedures in the production of foodstuffs of animal origin in order to obtain high-quality and safe products competitive on the world market"), record number 046009 III project, funded by the Ministry of Education and Science of the Republic of Serbia.

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EVALUATION OF BROWN SUGAR QUALITY FROM SUGAR BEET PROCESSING

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ABSTRACT: In the present study the quality of brown sugar from domestic sugar beet processing were investigated. It is either an unrefined or partially refined sugar consisting of sugar crystal with some residual molasses content, or it is produced by the addition of molasses to refined white sugar. At gobal sugar market various types of brown sugar are present. It can be produced from sugar cane or sugar beet, in principle according to three manufacturing processes. Depending on the region brown sugar have different names. In the dependence of origin and manufacturing processes it differs according to chemical composition, nutritive value, color, taste and grain size. Their popularity is due to the idea of consumers that brown-colored products are more natural, healthier and more valuable. Their employment in the food manufacture is based on aroma, color characteristics and nutritive value.
The aim of this paper was evaluation quality of brown sugar, produced in domestic sugar industry, from the aspect of food safety and nutritive value.
Key words: *brown sugar, quality, nutritive value*

INTRODUCTION

World sugar production is based on sugar cane (70 %) and sugar beet (30 %), while in the European Union 98% of total sugar production derived from sugar beet (Nolte and Grethe, 2011). Sugars have used in food production not only as a sweeteners but also as a valuable ingredients contributing to the taste, flavor, colour, energy, texture, nutritional value, hygroscopity, consistency, antioxidant effect, etc., of food (Clarke, 1995). Sugar always acts in more than one way, which accounts for its wide use in foods and beverages (Poel et al., 1998).
In sugar refining special crystal sugar products as brown sugar can be produced beside white sugar. A syrup layer is left around the crystals giving a special colour and flavor to the brown sugar. In principle, there are three manufacturing processes: a) evaporating crystallization of selected syrups b) mixing fine particle size white sugar with syrups, which are always of cane origin because of the flavour and c) cocrystallization method from special syrups. Depending on the origin: sugar cane or sugar beet and manufacturing processes, different types of brown sugar can be produced. Most producers make two or three types: light, medium and dark. The content of nonsugar substances increases with the intensity of the colour.
The aim of this study was to evaluate quality of brown sugar produced from sugar beet in domestic sugar industry.

MATERIAL AND METHODS

The investigations were conducted with samples of brown sugar produced from sugar beet in domestic sugar factory. Basic quality parameters of brown sugar, content of mineral matters and heavy metals were determined according to methods described in handbooks for the laboratory control of sugar processing (Milić et al., 1992). The methods are harmonised with

the regulations guided by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA, 2003). The obtained data were processed using a statistical software STATSOFT. Basic statistical descriptors were calculated (Hadživuković and Čobanović, 1994).

RESULTS AND DISCUSSION

In Table 1 average values and other statistical parameters of the quality parameters of the tested samples are presented in order to get a more realistic picture about the quality of brown sugar produced in our factories.
The results confirmed variation in the quality parameters of the examined samples which is understandable because sugar beet is a biological material whose quality depends on the growth conditions of sugar beet. The variations in certain parameters were also due to characteristics of processing conditions used in each factory. The largest coefficients of variation were observed in the content of sulphur-dioxide and reducing matters which implied to the fact that these parameters were largely dependent upon the processing conditions. Variations showed that sugar factories applied different processing conditions with different efficacy.

Table 1. Basic quality parameters of brown sugar

Parameter	Minimum	Maximum	Avarge	Standard deviation	Coefficient of variation
Polarisation (%)	98.77	99.58	99.13	0.336	0.339
Reducing matters (%)	0.027	0.150	0.081	0.040	48.971
Ash (%)	0.083	0.389	0.210	0.087	41.618
SO_2 (mg/kg)	2.650	7.969	5.323	2.459	46.202
Moisture (%)	0.106	0.288	0.197	0.065	32.960

Table 2 presents the composition of mineral matters in brown sugar. The values of single mineral matters varied widely, that were related to the different content of mother syrup - molasses in brown sugar crystals. Mineral matters in brown sugar have origin from heterogenous sugar beet material and conditions of technology.

Table 2. Mineral matters in brown sugar

Mineral matters (mg/kg)	Minimum	Maximum	Avarge	Standard deviation	Coefficient of variation
Potassium	102.38	461.59	219.04	128.03	58.45
Sodium	52.70	207.57	92.20	55.95	60.69
Calcium	155.80	967.73	436.78	360.13	82.45
Magnesium	2.21	3.59	2.84	0.55	19.28

The contribution of potassium, sodium, calcium and magnesium in ash are presented in Figure 1.
Table 3 presents Daily Values (DV) of nutrients for brown sugar which are calculated on the basis of using one cup of brown sugar daily, according The World's Healthiest Foods (Mateljan, 2007) and USD Nutritive Value of Foods (Gebhardt and Thomas, 2002).

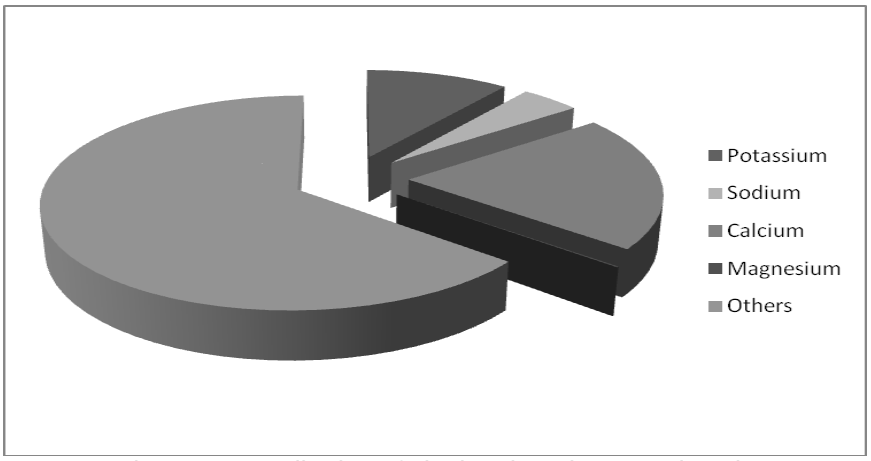

Figure 1. Contribution of single mineral matters in ash

Table 3. Contribution of mineral matters of brown sugar in human daily requirements

	Potassim	Sodium	Calcium	Magnesium
Daily requirements (mg)	3500	2400	1000	400
DV (%)	1.377	0.845	9.610	0.156

In the tested samples of brown sugar, the content of heavy metals (As, Pb, Cd and Hg) was determined and their frequency distribution is presented in Figure 2. The highest frequency distributions for arsenic, lead and coopper were in the concentration range 0.0 - 0.1 mg/kg, whereas 50% of samples had mercury concentration in the range 0.0-0.001 mg/kg.
The contents of heavy metals measured in the samples were all below the regulated limits for brown sugar (Sl.gl. RS br. 25/2010 i 28/2011) that confirming the health safety of brown sugar produced in Serbian sugar factories.

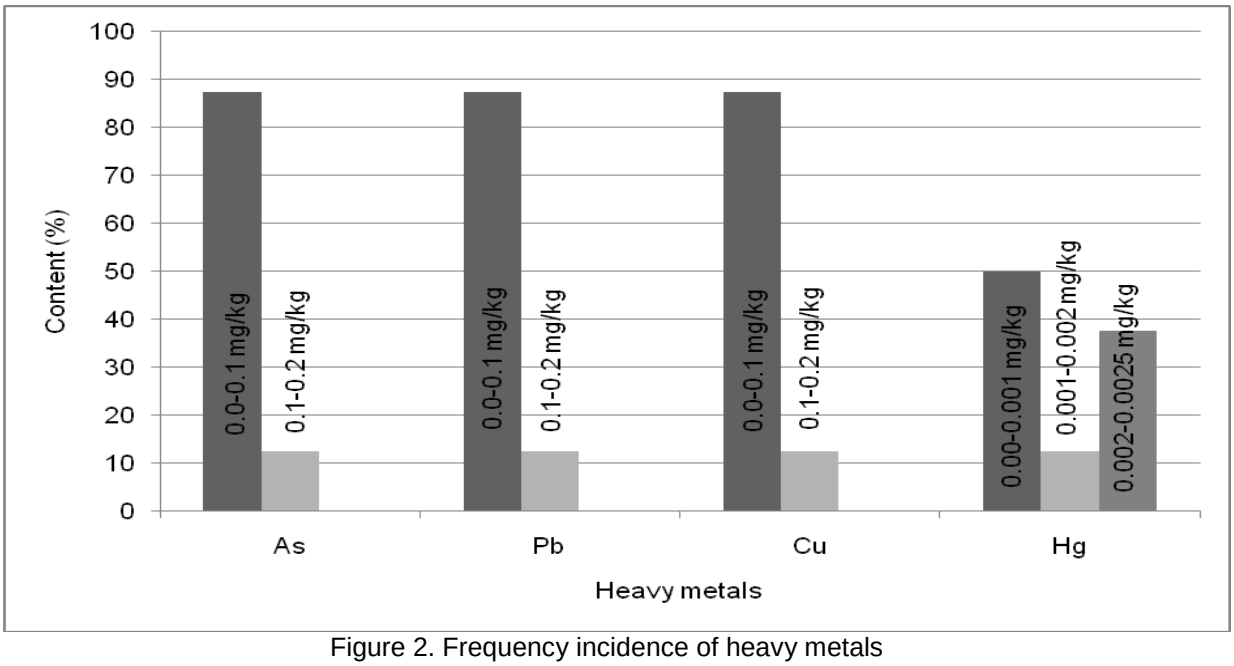

Figure 2. Frequency incidence of heavy metals

CONCLUSIONS

On the basis of the results it can be concluded that the investigated samples show the very different composition of brown sugar. The quality of sugar varies in the dependence of the sugar beet quality as raw material and the conditions of sugar beet processing. The mineral matters: potassium, sodium, calcium and magnesium present about 36 % of ash deterninated conductometrically. The content of nonsucrose compounds varies widely, that is related to the different content of mother syrup - molasses in brown sugar crystals. The content of heavy metals (As, Pb, Cu, Hg) confirm that brown sugar is health safety.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III 46005, financed by the Ministry of Science and Technological Development, Republic of Serbia.

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PROBABLE HEAVY METAL POLLUTION IN SEEDS AND POULTRY IN THE ENVIRONMENT OF CEMENT INDUSTRY OF ÇANAKKALE

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ABSTRACT: This study was carried out 18 villages and towns fronting to a cement factory in Mahmudiye village of Çanakkale district. Four poultry houses from each location was selected and 4 chickens, 4 eggs and the samples of seed wheat from each poultry houses were taken in winter, spring, summer and autumn seasons (January, April, July, October) of 2009 and 2010 years. One of the main aims of the study is to determine the level of selected heavy metals (Pb, Cd, Cu, Cr, Co, Mo and Ni) in the liver, legs and chests of the chickens fed near the places of cement factory. In the experimental period, from 2304 chickens, the samples of chicken liver, legs and chests, 2304 eggs and 576 seeds of wheat were collected. Heavy metal levels analyzed in the livers of the samples were found higher than the ones in the legs and chest samples (P≤0.01) and the heavy metal levels of the seed wheat collected in the poultry houses were found higher than the egg samples in the same poultry houses (P≤0.01). It has found that none of the heavy metals exceeded the limits determined by Turkish Food Codex and European Union.
Key Words: *Heavy metals, cement, poultry, wheat*.

INTRODUCTION

Today, the environmental problems are the most trouble some situations that threaten the natural balance and vibrant health. Environmental pollution which is the result of the developed industries, big powers ambition to earn more, people's and animal's food requirement, increase of population and efforts to get a civilized interviewee are becoming more important. Today, among the factors that cause environmental pollution the cement sector is usually the first thought. Research high lights the importance of cement sector as air pollutants. Indeed, the cement industry is one of the oldest branches of industry that process rather large amounts of solid material by a wide variety of equipments. The modern concrete roads, buildings, structures such as damns indicate that this industry has grown and developed in the past and in the present century. Environmental pollution also increased with the enlargement. The problems that caused by the sector are only limited by the effects of dust and the dust forms. However, in the last 30 years, noise, vibration and emissions of some gases such as sulfur dioxide and nitrogen oxides, an even discussions of possibility of heavy metals that could be in gases changed the size of the problems (Pekin, 2011). Cement factories damage residential areas and productive agricultural lands by the dust particles they emit and cause yield losses of products in those areas and change the natural chemistry of soil (Uysal et all., 2006). The pollution which is cause by cement factories also affect animal production at the same time. Toxic substances which are derived from the cement industry wastes are found in areas where the animals are pastured and particularly in water supplies. Heavy metals, toxic polluters, are durable for the enviromental situations, can effect the biological systems readily and give severe damages to the living organisms through food chains (Baş and Demet, 1992).
Second category of wastes, scrap tires, plastics, contaminated wastes, insecticides, bilge and composite materials are used as an alterntive fuel in the cement factory. Creation of heavy metals are taken place by using alternativ fuels in addtion to carbon-based primary fuels. In this research, the effects of pollutants (heavy metals), which are caused and released into the environment by the cement factory that is located 40 km south of Çanakkale, on feed sources (wheat) and poultries which are breeding in the area.

MATERIAL AND METHODS

The research was conducted in 18 separate locations which are located in the northern and southern directions of cement factory operating in Çanakkale's Mahmudiye town. 4 flocks are selected from each village. Four eggs, 4 chickens and wheat grains, which were used in feeding, were samples which were taken from each flock in 2009's and 2010's winter, spring, summer, autumn (January, April, July, October). 2304 chickens and eggs, 576 grain wheat samples are collected in testing period. The collected samples (leg, breast, liver, egg, wheat) are homogenized by the mixer and grinder. Subsequently, 5 g. each sample, was treated 25 mL of concentrated HNO3 (nitric acid) and 10mL of H_2O_2 (hydrogen peroxide) to in Erlenmayer flask, and burnt in the open area by the method of wet decomposition. Completely burnt samples were, with 10mL of distilled water. A control sample was prepared for each 10 samples. The temperature for wet decomposition in the open area was, set to 100^{o}C, the sample was held at this temperature for 1 hour, then after standing at 130^{o}C for 1 hour, and at 150^{o}C until the sample gets clarified. Each sample (breast, hindquarter, liver, egg and wheat) was measured for lead (Pb), cadmium (Cd), copper (Cu), chromium (Cr), molybdenum (Mo), cobalt (Co) and nickel (Ni) content. Statistical analysis has been done for, investigated years (2009 and 2010), seasons (spring, summer, autumn, winter), regions (nothern, southern and control settlements), Repeated measurement-ANOVA was used to investigate the effects of the amounts of heavy metal accumulated in chicken's hindquarter , liver and breast. Tukey's multiple comparison test was used for determination of different groups. (Winer et all., 1991; Zar, 1999). The statistical analysis was performed using Minitab and SPSS statistical package programme.

Findings

The evaluation of all obtained data has shawn, that the effects of organ x location interaction is very important for all heavy metals. (P=0.00). The amount of heavy metals which are accumulated in chickens' liver, chest and hindquarter, changes by location from where the test samples were received. (Figure 1).

Table 1. Accumulation of heavy metals in products by region.

		Area		
		North	South	Control
Heavy Metals mg/kg	Organ	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$
Pb	Leg	0.0360 ± 0.0009 ^{Ba}	0.0266 ± 0.0004 ^{Bb}	0.0278 ± 0.0005 ^{Bb}
	Liver	0.0650 ± 0.0017 ^{Aa}	0.0374 ± 0.0005 ^{Ac}	0.0404 ± 0.0005 ^{Ab}
	Breast	0.0191 ± 0.0004 ^{Ca}	0.0165 ± 0.0002 ^{Ca}	0.0138 ± 0.0003 ^{Ba}
Cd	Leg	0.0234 ± 0.0005 ^{Ba}	0.0238 ± 0.0003 ^{Ba}	0.0255 ± 0.0004 ^{Aba}
	Liver	0.0518 ± 0.0015 ^{Aa}	0.0338 ± 0.0003 ^{Ab}	0.0364 ± 0.0005 ^{Ab}
	Breast	0.0135 ± 0.0003 ^{Ca}	0.0150 ± 0.0002 ^{Ca}	0.0147 ± 0.0008 ^{Ba}
Cu	Leg	0.4176 ± 0.0068 ^{Ba}	0.5243 ± 0.0071 ^{Ba}	0.5039 ± 0.0200 ^{Ba}
	Liver	1.9630 ± 0.0264 ^{Ac}	2.2121 ± 0.0487 ^{Ab}	2.4896 ± 0.0827 ^{Aa}
	Breast	0.2326 ± 0.0038 ^{Ca}	0.3538 ± 0.0050 ^{Ca}	0.3353 ± 0.0158 ^{Ba}
Cr	Leg	0.2095 ± 0.0037 ^{Bb}	0.3119 ± 0.0050 ^{Ba}	0.0622 ± 0.0066 ^{ABc}
	Liver	0.3198 ± 0.0054 ^{Ab}	0.4775 ± 0.0079 ^{Aa}	0.0983 ± 0.0118 ^{Ac}
	Breast	0.1371 ± 0.0026 ^{Cb}	0.2103 ± 0.0038 ^{Ca}	0.0371 ± 0.0041 ^{Bc}
Co	Leg	0.0605 ± 0.0025 ^{Bb}	0.2118 ± 0.0061 ^{Ba}	0.0605 ± 0.0053 ^{ABb}
	Liver	0.0955 ± 0.0033 ^{Ab}	0.3377 ± 0.0091 ^{Aa}	0.0973 ± 0.0111 ^{Ab}
	Breast	0.0315 ± 0.0015 ^{Cb}	0.1379 ± 0.0045 ^{Ca}	0.0425 ± 0.0046 ^{Bb}
Mo	Leg	0.0707 ± 0.0028 ^{Bb}	0.2367 ± 0.0067 ^{Ba}	0.0575 ± 0.0051 ^{ABb}
	Liver	0.1135 ± 0.0054 ^{Ab}	0.3724 ± 0.0096 ^{Aa}	0.0832 ± 0.0072 ^{Ac}
	Breast	0.0373 ± 0.0017 ^{Cb}	0.1555 ± 0.0047 ^{Ca}	0.0396 ± 0.0040 ^{Bb}
Ni	Leg	0.0430 ± 0.0010 ^{Bb}	0.0554 ± 0.0022 ^{Ba}	0.0276 ± 0.0008 ^{Bc}
	Liver	0.0759 ± 0.0023 ^{Ab}	0.0804 ± 0.0031 ^{Aa}	0.0429 ± 0.0012 ^{Ac}
	Breast	0.0223 ± 0.0007 ^{Cb}	0.0348 ± 0.0014 ^{Ca}	0.0157 ± 0.0006 ^{Bc}

** In the same area, differences between the different organs shown in capital letters are important. (P≤0,01).*

*** Same organ, differences between the different regions shown in small letters are important. (P≤0,01).*

Among the samples gained from the North and South settlements, the most density of heavy metals has been found in liver tissues, the lowest amount has been observed in chest samples. Similar findings were also detected in samples obtained from control areas, the most intense accumulation observed was in liver, and the lowest accumulation was observed in leg and breast. Of all investigated heavy metals, Cu was determined at the highest level in the liver (Figure 1).

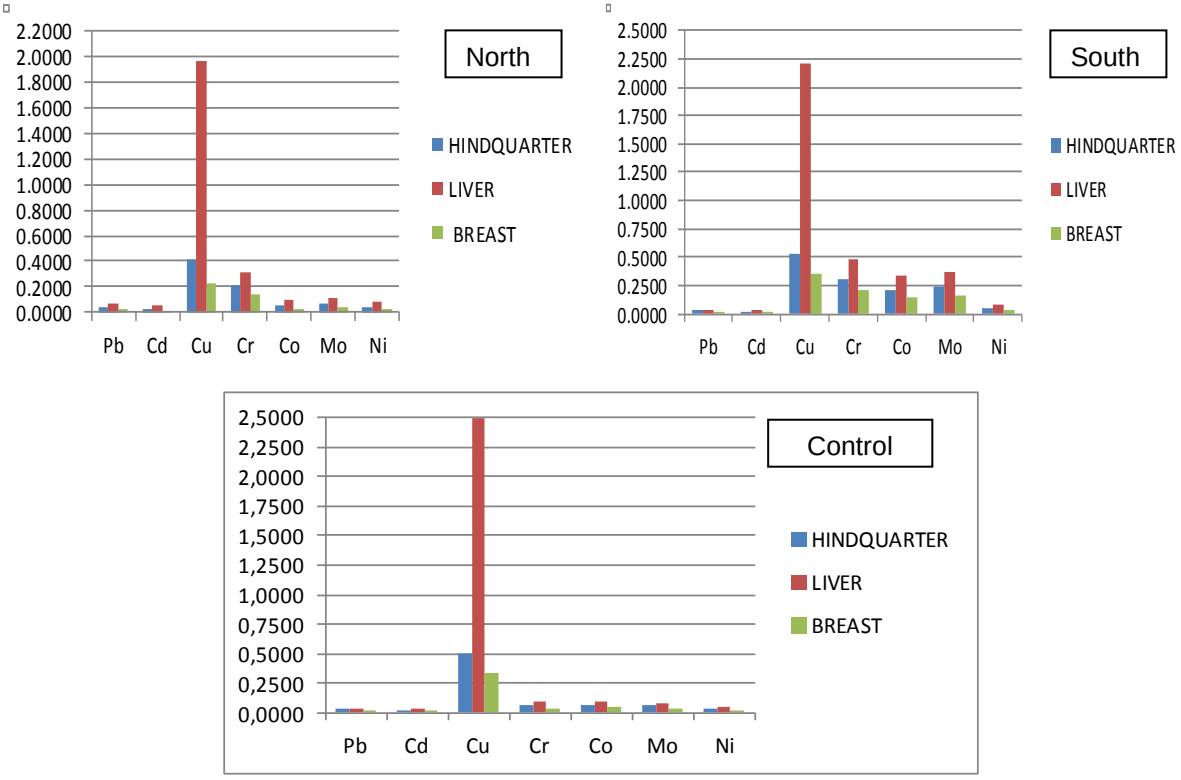

Figure 1: Averages of heavy metal accumulation (ppm) in organs fromin north, south and control groups.

Pb is significantly higher in northern settlements than in southern and control settlements in all analyzed tissue samples. It was determined that there is a significant difference between south and control settlements. Analyses of egg and wheat samples show that some heavy metal contaminations decreases by increasing the distance from the factory. However, when Pb, Cu and Cr are analyzed it is determined that although in some areas accumulation was under the legal limits, it is still high. Level of heavy metals in wheat used as feed for poultry is determined as higher than the level in eggs from same poultry ($P \leq 0.01$). The determined were below the tolerance limits set by Turkish Food Codex (TGK) and EU regulations. In TGK, tolerance limit has been specified for Pb in poultry meat at 0.1 ppm, edible poultry offals (liver, hearth, gizzard) at 0.5 ppm, wheat at 0.2 ppm and for Cd in poultry meat at 0.05 ppm, poultry liver of poultries at 0.5 ppm, wheat grain at 0.1 ppm. This condition indicates that the situation is not harmful for human and animal health in the light of our datas that are identified from accumulation of heavy metals. Provided research findings indicates that analyzed accumulation of heavy metals decrease in the following order: liver > leg > breast. This finding is consistent with similar literature datas. Literature datas report that Pb is a toxic metal that affects bone linearly. Accumulation of heavy metal in bone tissue has not been studied, because the research was focused on edible, tissues, organs and grain used as feed. However, it is known that %90-95 % of lead present in that held in the body is accumulated in bones. (Finley, 1978; Ozan, 1996). This is followed by liver and kidneys (Humphereys, 1991; Roberts et. all , 1978).

DISCUSSION AND CONCLUSION

Animals exposure to metals usually take place through water and feed. However, it is very difficult to identify the environmental problems caused by metals even under today's technology. Confidence limits for heavy metal concentrations in these sources must be known. Especiallly Cu, Pb, Cd, Ni metals causes animals to have acute and chronic toxicity. (WHO, 1987). During the research, it has not been seen any intoxication that can lead to acute poisoning. While acute poisoning can easily be recognized, chronic poisoning is usually hiddesn. Chronic poisoning causes deterioration in terms of quality and quantity of production, thus causes economic losses. Chronic poisoning can be diagnosed in animal by analysis of milk, blood, hair, wool, liver, kidneys and carcasses. (WHO,1987). Although some heavy metals and trace elements are anatomical components for live biological systems taken from the environment; when they are taken in overdose or chronic levels, it may poison animals or people. Irwin et all. (1989), reported that lead accumulated in descending order bone, kidney, liver and muscle in ducks fed for 14 weeks in an artifical pond that has different amounts of lead particles. In samples obtained from the northern areas studied in this research, detection of accumiulation of Pb at relatively high level from examined tissues are similar to the findings of the other studies. Although our findings indicate that accumulation of Cd, especially in liver, is much higher in the nothern region samples, they are not present at toxic levels.

The findings of our study show that Pb levels are higher in the northern areas, but lower in the southern areas. ($P \leq 0.01$). In a similar study, Kurnaz (2008) determined that heavy metal accumulation in the liver is higher than in the hindquarter and it is appropriate for TGK and the European Union notification. This result supports our findings. Exhaust fumes that are carryied by prevailing winds are concentrated in the northern areas more than in the areas where the cement plants are located. This situation can be explained by atmospheric transport of Pb and closeness of İzmir-Çanakkale highway. It is known that living organisms can continue to grow in an environment where heavy metals can be found, but they do not have a mechanism to permanently eliminate the toxic effects. From this point of the research findings, liver, leg, breast, egg and, wheat grain do not affect consumers health in a negative way.

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CHARACTERIZATION OF BULGARIAN COMMON WINTER WHEAT VARIETIES BASED OF THE GRAIN HARDNESS

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ABSTRACT Grain hardness is a basic differentiating element of wheat standard classifications. The hardness of the wheat endosperm is genetically determined. That is considered as a stable variety's index. The attention that is paid to the hardness as an indicator of the quality is due to the different technological relation of the wheat. Twenty four Bulgarian varieties of common winter wheat, developed over a very long period of time (60 years) at Institute of IRGP-Sadovo were studied. Using Inframat 8600-H, grain hardness, vitreousness and content of crude protein, were determined during 3 crop years. Statistically it has been proven that old varieties like Jubileina 3 and No. 301, as well as Katiya, Lada, Murgavets and Guinness, are wheat with hard endosperm and high vitreousness, but the old variety Okerman and varieties like Sadovo super, Mustang, Prelom and Diamond have soft endosperm and therefore are low vitreous. The rest of the studied varieties have medium hard to hard endosperm.Using analysis of variance it has been proven that under certain agro-climatic conditions the index varies during 3 years of study: F exp.=7.76 > F crit.=3.12
Key words: *wheat variety, grain hardness, vitreousness, crude protein*

INTRODUCTION

The utmost attention paid to hardness as a grain quality trait is due to the different technological handling of wheat having different structural-mechanical properties. It is considered that endosperm hardness is genetically determined and appears a sustainable variety feature.

The better knowledge of the physico-chemical mechanism and the endosperm genetic control helps towards breeding, marketing and utilization of the end-use product. According to Symes (1965) the conversation of hard wheat to soft wheat or vice versa can be achieved by backcrossing. The grain hardness of new wheat will be influence both by the hardness of the donor parent and by the degree to which modifying genes are carried over. This demonstration of the simple inheritance of grain hardness measured by particle size index is significant to wheat improvement programs.

It has been established through a scanning microscope that hardness reflects total cell structure, the protein matrix type, and mostly the protein-starch relation, in this way characterizing the endosperm type (Belcheva, 1993).

Friabiline is a specific endosperm protein associated with starch granules of wheat grain. Chemically these are various polypeptides, primarily puroindolines. Hardness (Ha) locus of chromosome 5DS makes the distinction between hard and soft classes of wheat. (Pasha et al., 2010).

Hard grain wheat and soft grain wheat have different behaviour when ground and sieved, and also take different energy consumption. The difference in the flour microstructure determines their rheological properties. Hard grain flour is sharp and easily sieved. Soft grain flour, on the other hand, is fine, easily adheres, hard to sieve and glide. This is due to relatively smaller particle size, relatively larger surface of the flour, and the greater particle cohesion (Zwingelberg, 1983).

Based on the hardness-protein relation, Moss (1973) suggested the following wheat grading: common soft with 10-11% protein content; common hard with 12-14.5% protein content.

However, the studies of some American and Australian authors on the protein-hardness relation do not establish a relation between these two features.
The studies of Shikrenov at al., (1967) refer to the varieties spread in Bulgaria in the 60s. Stoeva at al., (1988) using Brabender instrument have characterized only some wheat varieties created at Dobrudja Agricultural Institute, near Gen. Toshevo in the 80 s, that were referred to the wheat having hard to medium hard endosperm. The group of varieties studied by Belcheva et al., (1990) includes a few varieties created at the IPGR, Sadovo. Mangova et al., (2004, 2006) were evaluated chiefly hybrid - mutant wheat lines.
The present study comprises a large number of wheat varieties created at IPGR, Sadovo over the long period of 60 years.

MATERIAL AND METHODS

The objects of the present study were 24 common winter wheat varieties (T. Aestivum), created over a 60-year-long period of time at Institute of Plant Genetic Resources, Sadovo.
The following grain quality parameters have been determined over a period of three consecutive crop years: endosperm hardness and crude protein content by Inframat 8600-H and total grain vitreousness by farinotom.

RESULTS AND DISCUSSION

The grain hardness for the three crop years was within the range of 48.0-57.67, and the mean was 54.42 (Table 1). It was statistically proven that the varieties with the hardest grain endosperm were: Katiya, Pobeda, Jubileina 3, Lada, Guinness, and Murgavets. It was also statistically proven that the following varieties have the softest endosperm in descending order: Sadovo super, Diamand, Okerman, Prelom, Mustang and Zdravko. The rest of the studied varieties have medium hard to hard endosperm.

Table 1.Estimation of wheat varieties according to grain hardness

Variety	Range	Average value	Deviation	Significance
№ 301	8	56.33	1.92	*
Pobeda	2	57.33	2.92	+ +
Okerman	22	50.0	-4.42	- - -
Momchil	11	55.33	0.92	*
Jubileina 3	3	57.33	2.92	+ +
Zdravko	19	52.0	-2.42	-
Murgavets	6	56.67	2.25	+
Lada	4	57.33	2.92	+ +
Geiya -1	12	55.0	0.58	*
Yunak	15	55.0	0.58	*
Guines	5	57.0	2.58	+
Sadovo 772	10	56.0	1.58	*
Mustang	20	51.0	-3.42	- -
Liusil	16	54.33	-0.08	*
Prelom	21	50.67	-3.75	- -
Sadovo 552	17	54.33	-0.08	*
Bononiya	18	53.0	-1.42	*
Katiya	1	53.67	3.25	+ +
Sadovo 1	13	55.0	0.58	*
Sadovo super	24	48.0	-6.42	- - -
Diamant	23	49.0	-5.42	- - -
Sadovska beliya	9	56.33	1.92	*
Boriyana	14	55.0	0.58	*
Petiya	7	56.33	1.92	*

The average grain protein content over a three-year period was 12.5% (Table 2).The highest in protein content were the hard grain varieties Jubileina 3-13.37%, № 301- 13,17%, Pobeda 13% and Sadovo 5.52 -13.3%. The soft grain varieties: Sadovo super, Prelom, Okerman and Diamand were in close range in protein content of 12.2 %-12.8%. However, according to Moss H.J. (1973) and Bushuk W.'s (1986) classification, this is the range of protein content in hard grain varieties. Therefore Bulgarian soft endosperm varieties were characterized by higher protein content, which is not typical of this group. Mc Ritchie (1980) referred to these wheat as exceptions. This resulted from the Bulgarian breeding programs, whose priority has been the protein content without controlling the endosperm hardness, because the requirements for technological qualities parameters to recognizing a new varieties, is 12% minimum protein content.

Table 2. Estimation of wheat varieties according to crude protein content

Variety	Range	Average value %	Deviation	Significance
№ 301	3	13.17	0.69	+
Pobeda	4	13.00	0.53	*
Okerman	5	12.80	0.33	*
Momchil	7	12.77	0.29	*
Jubileina 3	1	13.37	0.89	+ +
Zdravko	22	11.97	-0.51	*
Murgavets	21	12.03	-0.44	*
Lada	20	12.13	-0.34	*
Geiya -1	23	11.87	-0.61	-
Yunak	18	12.27	-0.21	*
Guines	8	12.63	0.16	*
Sadovo 772	15	12.30	-0.17	*
Mustang	11	12.50	0.03	*
Liusil	9	12.57	0.09	*
Prelom	6	12.77	0.29	*
Sadovo 552	2	13.30	0.83	+ +
Bononiya	12	12.50	0.03	*
Katiya	17	12.27	-0.21	*
Sadovo 1	13	12.40	-0.07	*
Sadovo super	10	12.57	0.09	*
Diamant	19	12.20	-0.27	*
Sadovska beliya	24	11.33	-1.14	- - -
Boriyana	14	12.37	-0.11	*
Petiya	16	12.30	-0.17	*

Dependency of grain hardness - protein content varied within varieties and years. The correlation coefficients were low and statistically not significant, so this cannot serve as a basis to grade the wheat varieties.

According to Mc Ritchie (1980) the common wheat varieties with hard endosperm were characterized as vitreous, whereas the common wheat varieties with soft endosperm as non vitreous. However, some Russian authors' studies present data also on high vitreous varieties with soft endosperm.

The total virtuousness of grain for the three years was within the range of 42.33%-69%, and the mean was 57.78% (Table 3). The old hard grain variety No 301 showed maximum value of 69%, quality standard Pobeda 67% and Murgavets 64%. Sadovo 552 variety is an exception, being high vitreousness wheat 63.3%, but in hardness it ranks 17th (54.33) among the 24 varieties studied.

The soft endosperm wheat, like the old variety Okerman 42.33%, Zdravko 46.67%, Sadovo super 49.67% and Diamant 48% have a well established non vitreous endosperm. It should be noted, that also in the two groups of varieties with hard and soft endosperm, the total vitreousness varies within relatively broad range.

Table 3. Estimation of wheat varieties according to grain vitreousness

Variety	Range	Average value%	Deviation	Significance
№ 301	1	69.0	11.22	+ +
Pobeda	2	67.00	9.22	+
Okerman	24	42.33	-15.44	- - -
Momchil	10	60.33	2.56	*
Jubileina 3	8	62.33	4.56	*
Zdravko	23	46.67	-11.11	- -
Murgavets	3	64.00	6.22	*
Lada	6	62.33	4.56	*
Geiya -1	19	54.33	-3.44	*
Yunak	11	60.33	2.56	*
Guines	7	62.33	4.56	*
Sadovo 772	12	59.67	1.89	*
Mustang	21	49.33	-8.44	-
Liusil	13	58.67	0.89	*
Prelom	14	57.67	-0.11	*
Sadovo 552	4	63.33	5.56	*
Bononiya	18	54.67	-3.11	*
Katiya	9	61.33	3.56	*
Sadovo 1	15	57.33	-0.44	*
Sadovo super	20	49.67	-8.11	-
Diamant	22	48.00	-9.78	-
Sadovska beliya	16	57.33	-0.44	*
Boriyana	17	56.00	-1.78	*
Petiya	5	62.67	4.89	*

The dependence between the two parameters- total vitreousness and grain hardness in years, expressed by correlation coefficients, was low to medium high: for 1st year r = 0.51, for the year 2nd r = 0.83, for the 3rd year r = 0.59.

The evaluation of the Bulgarian common winter wheat varieties indicates that the endosperm hardness varies, which requires testing whether or not agro-climatic conditions have affected over the parameter values. Tthrough analysis of variance it was found that abiotic factors statistically significant have affected the grain hardness F exper. = 7.76 > F crit. = 3.12.

CONCLUSIONS

The studied Bulgarian winter wheat varieties were referred to two classification groups according to grain hardness: varieties with hard and medium hard endosperm, and varieties with soft endosperm.

The varieties with the hardest grain endosperm were: Katiya, Pobeda, Jubileina 3, Lada, Guinness and Murgavets. The varieties with the softest endosperm in descending order were: Sadovo super, Diamand, Okerman, Prelom, Mustang and Zdravko.

The correlation coefficients of the dependence grain hardness – crude protein content were low and statistically not significant.

It was found that environmental abiotic factors statistically significant have affected the grain hardness.

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DETERMINATION OF TOTAL PHOSPHORUS IN THE MEAT PRODUCTS

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ABSTRACT: Phosphate and polyphosphate additives are added to meat and other food products because they serve as an emulsifier of fat, water and protein, and stimulate water binding. Phosphates affect the texture of the product, prevent discoloration, improve emulgation of fat and improve flavor, prevent oxidation of unsaturated fatty acids and affect the pH value. Existing legislation defines the maximum amount of phosphorus (expressed as P_2O_5), that can be added to meat products and which is 5 g/kg. In the Sanitary Chemistry department of Public Health Institute, the content of phosphorus in these products in the samples presented for analysis is determined on a daily basis. In the period from May 2010 to November 2011 there were 85 samples of meat products of ungulates and poultry analised for content of total phosphorus. The content of total phosphorus, g/kg, expressed as P_2O_5 was determined using the standard method "Meat and meat products – Determination of total phosphorus content – spectrophotometric method," JUS ISO 13730, 1999. The principle of the method is based on dry ashing and acid ash hydrolysis using nitric acid, followed by addition of ammonium monovanadate and ammonium heptamolybdate causing the creation of yellow colored compound, whose intensity is measured spectrophotometrically at 430 nm. Providing assurance in the results of testing laboratory secures through internal quality control (using the control map; Nordtest method) and frequent participation in inter-laboratory comparative tests. Of the total number of samples tested 24 samples or 28.2% had a phosphorus content (expressed as P_2O_5) greater than 5 g /kg, while in 47 samples or 55.3% phosphorus content ranged from 4 g/kg to 5 g/kg.
Key words: *phosphorus, meat products*

INTRODUCTION

Phosphorus is an essential element indispensible for normal functioning of both human and animal organism. Most of the phosphorus in an organism is in the form of phosphate and 85 percent of it out of total phosphorus content is found in the bones. Phosphorus is an important component of phospholipids that build cell membranes, then the energy-rich compounds such as adenosine triphosphate and creatine phosphate and plays an important role in maintaining acid-base balance (Stamenković, 2006).

Apart from natural phosphorus which originates from meat, in the meat products there is also phosphorus originating from additives, among which phosphates, as well as some soy-based products are specifically represented. Among water binding agents in meat, phosphates are of greater practical significance in meat processing. When added to chilled meat, in which natural phosphates (ATP) have been spent, phosphates renew their good water binding capacity which prerigoral meat had. Apart from its effect on water binding capacity, the phosphates have an impact on the texture of the product, prevent discoloration, improve emulgation of fat, improve taste, prevent oxidation of unsaturated fatty acids and affect pH values. The dose of phosphates is commonly determined according to the quantity of meat and fatty tissue in the product. It is believed that the technologically optimal quantity of phosphate added is approximately 3 g/kg, and that it should not be greater than 5 g/kg. Phosphates added in larger quantities are not contributing to product's quality, but reduce the water binding capacity, have prooxidative effect and give those products astringent, soapy or bitter taste (Saičić et al., 2008). Certain phosphates create complexes with ions of iron (Fe) and copper (Cu), which are known catalysts for oxidative process, and in this way prevent fat and meat rancidity. Thus the phosphate products are also considered as having the

properties of preventing oxidative changes, so as delaying the prooxidative effect of table salt (Stamenković, 2006).
Apart from colors and sweeteners in food, the use of food additives as the food additive category in various types of food has been regulated by the Regulation on use of food additives, which prescribes specific criteria of purity, as well as other requirements which must be met in production and trade. According to the mentioned Regulation, the maximum quantity of phosphates expressed as P_2O_5 that can be added to meat products singly or in combination is 5 g/kg (Pravilnik o upotrebi prehrambenih aditiva, osim boja i zaslađivača u hrani, 2008).
The Regulations on the quality of meat products and the Regulations on the quality of poultry meat products still have not been in compliance with the Regulations on use of food additives, except for colors and sweeteners in food, as it is done in neighboring countries. By regulations mentioned, the total phosphates content in final product expressed as P_2O_5 should be indicated as larger than the content that can be added i.e. over 5 g/kg (Pravilnik o kvalitetu proizvoda od mesa, 1974 i Pravilnik o kvalitetu proizvoda od mesa pernate živine, 1991).

MATERIALS AND METHODS

In the period from May 2010 until November 2011 were examined 85 samples of meat products of ungulates and poultry meat for content of total phosphorus. The analyzed products, originally from domestic and foreign manufacturers, were from domestic market, delivered to the laboratory.
Total phosphorus content, g/kg, expressed as P_2O_5 was determined by standard method of “Meat and meat products – Determination of total phosphorus content – Spectrophotometric method” JUS ISO 13 730 from 1999.
This method is based on a dry ashing and an acid ash hydrolysis using nitric acid, followed by the addition of ammonium monovanadate and ammonium heptamolydate which results in a formation of a yellow color compound, intensity of which is measured spectrophotometrically at a wavelength of 430 nm (JUS ISO 13730, 1999). Measurments were performed on UV-VIS spectrophometer Cary 1, Varian.
Measurement uncertainty was performed using Nordtest method which consists of two components. The first component is the reproducibility of the method as a result of laboratory performing a minimum of 20 "in house" double tests of reference sample by the same technician and the same chemical analyst on different days. The second component represents the accuracy or bias, which was taken from the reports from interlaboratory competition in which our laboratory participated. The obtained uncertainty (with coverage factor k = 2) was 10%.
As a confirmation of achieving good results, our laboratory successfully participated in interlaboratory competitions, with good results. Within the project "Strengthening BiH Accreditation System" in 2010 by The Institute for Accreditation of Bosnia and Herzegovina, an analysis of autoclaved freshly chopped fish was performed in organization LIVSMEDELS VERKET The National Food Agency of Sweden, and the analysis of pate within the "Interlaboratory comparative foodstuffs testing" in organization of Pharmaceutical Society of Serbia, Division of Sanitary Chemistry, Belgrade.

RESULTS AND DISCUSSION

Content, measured by spectrophotometry, total phosphorus (as P_2O_5) in 85 meat products is shown in Table 1.

Table 1. The content of phosphate (as P_2O_5) in the analyzed samples

Phosphate content as P_2O_5 (g/kg)	Number of samples	Percent
1-2	3	3,5
2-3	4	4,7
3-4	7	8,2
4-5	47	55,3
5-6	12	14,1
6-7	12	14,1
IN TOTAL	85	100

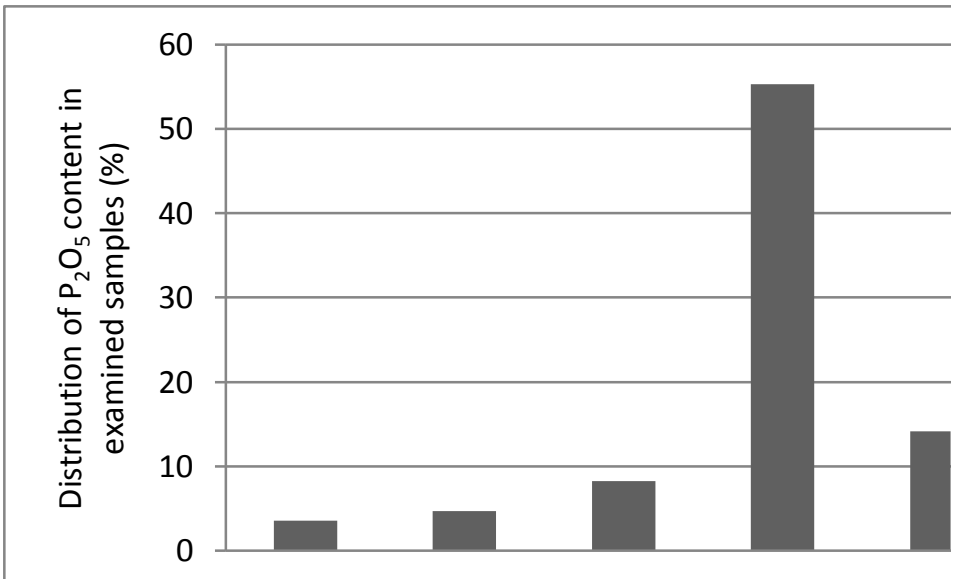

Figure 1. Distribution of P_2O_5, content in examined samples (g/kg)

Higher content of phosphate in meat products does not necessarily have to be the consequence of adding more phosphates, i.e. exceeding the maximum of permitted amounts added. To higher content of phosphorus can contribute not only basic constituents (high quality meat), but also other components which constitute the meat product of either vegetarian or animal origin. High quality meat is rich in proteins, and thus in phosphorus, which has an impact on higher phosphorus content in the product.

To determine the content of "added phosphates" by the standard method (ISO 13730/99), one should determine the total phosphorus content first, and then subtract from it the content of natural phosphorus, obtained by multiplying the protein content of the product by the number 0.0106, which represents the content of phosphorus in 1% meat proteins. Using this method we get reliable results with "clean" meat products, such as "cooked ham", "cooked shank" and "cooked chops", in proteins of which we find a relatively constant amount of phosphorus. However, in products which in addition to meat contain fat and connective tissues, liver, blood, mechanically separated meat from bone, protein products, cheese and other foods, by applying this method it is not always possible to get accurate values for "added phosphate" content (Vuković and Milanović-Stevanović, 2005).

This paper presents the results of a content of total phosphate, i.e., without subtraction of natural phosphorus content. Also, the meat products are not sorted into groups, because of shortcomings of existing legislation. What should be particularly emphasized is that, currently, quality control of these products is estimated on the basis of SFRJ regulations which are still in force (Pravilnik o kvalitetu proizvoda od mesa, 1974 i Pravilnik o kvalitetu proizvoda od mesa pernate živine, 1991) which are still in force and not in compliance with the new regulations on the use of additives (Pravilnik o upotrebi prehrambenih aditiva, osim boja i zaslađivača u hrani, 2008).

CONCLUSION

At the highest percentage of tested samples, the content of total phosphorus ranged from 4-5 g/kg or 55.3% of samples. Over 5 g/kg was measured in 24 samples, and only in 14 samples the total phosphate content was below 5 g / kg.
There were no samples containing over 7g /kg phosphates, which means that all the samples were in accordance with the maximum permissible amount from future Regulations on quality control of these products (which will limit the phosphate content in these products to 7 g / kg) and that they were manufactured in accordance with good manufacturing practice (GMP).

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DETERMINATION OF METHIDATHION IN BARLEY MALT BY A SOLID PHASE EXTRACTION METHOD

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ABSTRACT: The use of pesticides in agriculture has brought many obvious benefits, but their inappropriate use can result in unacceptably high levels of these compounds in cereals. Pesticide residues can be found even when they are applied in accordance with good agricultural practices. A solid phase extraction (SPE) method has been developed for determination of methidathion in barley malt samples. The method involves a rapid extraction procedure with acetonitrile using salting out step. Non-polar coextractives are removed by passing a portion of the acetonitrile extract through an octadecyl (C_{18}) solid phase extraction clean-up cartridge. An aliquot is taken and concentrated for second extract clean-up, which is done with a carbon cartridge coupled to an aminopropyl cartridge using acetonitrile: toluene 3:1 (v/v) as the elution solvent. Analysis is performed by gas chromatography, employing mass selective detection in the selected ion monitoring mode. The method rendered recoveries ranging between 91 and 96%, the associated relative standard deviations ranging between 1 and 3%. Limit of detection methidathion were less than or equal to 0.002 mg/kg. The applicability of the proposed method to detect and quantify pesticide residues has been demonstrated by the analysis of 20 genuine samples. The methidathion content in barley malt samples is not regulated by Serbian legislation (Official Gazette of RS, 28/11) and relevant EU regulation (Council Directive 90/642/EEC, 1990). The methidathion concentration found in barley malt samples were bellow the MAC value permitted by EU regulations and in accordance with the currently valid Serbian regulations for barley.
Key words: *barley malt, methidathion, solid phase extraction, residue analysis*

INTRODUCTION

Barley (*Hordeum vulgare* L.) was originally used as human food but, with the rise in prominence of wheat and rice, its use was altered to animal feed and malting. Presently, about two-thirds of the barley crops are used as feed, one-third for malting and only about 2% for human food (Baik et al., 2004). A high-protein form of malting barley is often an ingredient in blended flours and it is typically used in the preparation of yeast breads and other baked goods (Evers et al., 1994). In view of the present knowledge, barley deserves to be incorporated into various food products on account of its potential heath benefits.
Because of high contents of starch and storage proteins, barley grains are an attractive target for insects and microbial pathogens. The vulnerability of the grain to insect and pathogen attacks is expected to increase during storage when amino acids, fermentable carbohydrates, various nitrogen forms and other degradation products accumulate as reserve polymers in the starchy endosperm (Fincher and Stone, 1993). The increased dependence of successful storage of cereal grains on pesticides has invited concerns about pesticide residues and their effects. It has lead to the development of numerous multiresidue methods as the most advantageous approach to residue analysis. Screening for traces of pesticides in cereals can be an extremely challenging task due to large quantities of co-extracted components (e.g., starch, proteins) which may adversely affect the method and instrument performance. The analytical methods for assessment of pesticide residues in cereal samples require an initial sample preparation step in which the analytes of interest are isolated from the bulk of grain matrix. Some methods may require subsequent steps to further purify the sample extract and to concentrate the analytes for final determination step.

An effective clean-up step is required in order to eliminate interfering compounds from the extract. The trend in the development of methods for sample preparation has been towards reducing solvent volume and the time required for conventional methods such as Soxhlet extraction. Miyake et al. investigated the transformation of agrochemical residues from barley to malt at various stages of the malting process (Miyake et al., 2002). The degree of dissipation of the residue pesticides due to volatilization and formation of breakdown products depends greatly on moisture content, storage temperature and the type of cereal stored (Desmarchelier, 1978; Holland et al., 1994). Studies were conducted on the persistence potential of pesticide residues in grains subject to long-term storage (Hadjidemetriou, 1990; Lalah and Wandiga, 2002). The carryover of pesticide residues to malt was found to depend on the log P_{ow} (partition coefficient between n-octanol and water) values of the active substance.
Methidathion (S-2, 3-dihydro-5-methoxy-2-oxo-1,3,4thiadiazol-3-ylmethyl-O,O-dimethyl-phosphor-odiththioate) is a thioester of the thiophosphoric acid. It belongs to the group of organophosphorus non-systemic insecticides and acaricides (Figure 1) which was commercially available since 1966. It is widely used to control sucking and chewing insects and spider mites on many crops such as corn, fruits, vegetables, tobacco, cotton and sunflowers in the field and stored raw agricultural commodities on account of its low persistence and high effectiveness. Methidathion is declared as highly toxic for humans (Richardson and Gangolli, 1993) as well as birds, bees and aquatic organisms (Hartley and Kidd, 1987). Methidathion is not included among the active substances in Annex I to Directive 91/414/EEC and it is banned from the market in the Republic of Serbia (http://ec.europa.eu/sanco_pesticides).

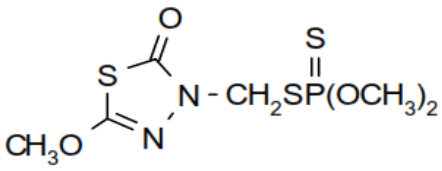

Figure 1. Structure of methidathion

The aim of this work was to determine methidathion residues in barley malt. The monitoring of methidathion residues in barley malt is commonly performed by gas chromatography (GC). Due to fairly common availability of the instrumentation, simplicity of procedures, speed, precision and accuracy, gas chromatography methods enjoy wide popularity. Residues were determined using a gas chromatograph equipped with a mass selective detector (MSD).

MATERIAL AND METHODS

Chemicals and solutions

The analytical standard of methidathion (98.2%) was purchased from Dr. Ehrenstorfer GmbH (Germany). The stock standard solution was prepared in methanol (J.T. Baker, USA) for GC-MS. Calibration standards were prepared by diluting the stock standard solution in methanol. Working standard solutions methidathion were used to calibrate the GC/MS system and spike samples barley malt in recovery experiments. All the solvents were of pesticide residue analysis grade (J.T.Baker, USA). Anhydrous sodium sulfate and sodium chloride (J.T. Baker, USA) were also used.

Sample preparation

Methidathion was extracted from homogenized samples of barley malt (10 g) by mechanical shaking with 20 ml acetonitrile and addition of sodium chloride. The extraction was repeated twice, each time with 20 ml acetonitrile. An advantage of extraction with acetonitrile rather than with other solvents is that it easily separates pesticides when salt is added to the sample.

When combined with salt, acetonitrile provides a well-defined phase separation, it renders unnecessary the use of hazardous non-polar organic solvents, and it achieves high pesticide recoveries (Lazić et al., 2008, Lazić et al., 2009, Ostojić et al., 2009).
Clean-up is necessary in order to reduce the detection limits of the method and/or to avoid interferences from the barley malt matrix. The concentrated sample extracts may contain a high content of co-extractives which can damage the capillary GC column, resulting in a matrix enhancement effect (Hajšlová et. al, 1998). Solid-phase extraction was carried out using C_{18} column packed with 500 mg of highly cross-linked octadecyl sorbent. The extraction columns were conditioned by passing 6 ml of acetonitrile through them. The sorbent was never allowed to dry during the conditioning and sample loading steps. Exactly 4 ml of barley malt extract (equivalent to 2 g sample) was transferred to the column. Anhydrous sodium sulfate was added to the previously collected eluates in order to remove water from them. Afterwards, a 2 ml (equivalent to 1 g sample) aliquot of the eluates was transferred to ENVI Carb column (6 mL, 500 mg) coupled to an aminopropyl column (6 ml, 500 mg). Methidathion residues were eluted from the column by a 20 ml mixture of acetonitrile and toluene (3:1). The eluent was collected in a 50 ml round-bottom flask and concentrated to about 2 ml in an evaporator with a water bath at 35ºC. Ten ml acetone was added to the flask and the flask was re-evaporated to dryness. After that, 10 mL portions of acetone were added two times and evaporated to low volume after each addition. The entire extract was carefully transferred to 2 mL GC vials which were then ready for injection.

GC–MS analysis
Determinations were performed on an Agilent Technology 6890 N gas chromatograph fitted with an HP5975 B mass selective detector and HP-5MS column. The gas chromatograph was equipped with CTC CombiPAL sampler and split/splitless injector with electronic pressure control. The mass spectrometer was used with electron impact ionization (70 eV) in Scan mode (35-500) and selected ion monitoring (SIM) mode for ions 145, 85, 93, 125 (m/z) as presented in Figure 2. The ion source and MS Quad temperatures were 230 and 150ºC, respectively. We used a fused silica capillary column 30 m x 0.25 mm I.D., 0.25 µm (bonded 5% phenyl, 95% dimethylpolysiloxane) supplied by Agilent Technologies.

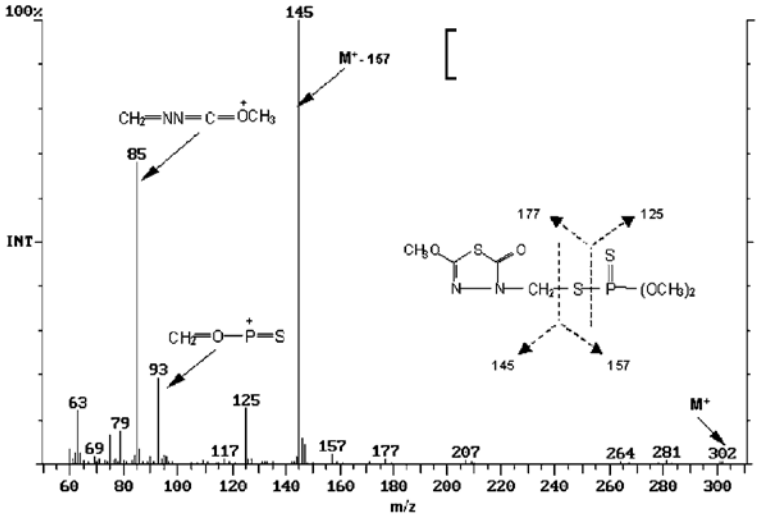

Figure 2. Mass spectar of methidathion

The pulsed splitless injection (pressure pulse 21.80 psi, pulse time 0.5 min, injection volume 1 µl, syringe size 10 µl) was done. The oven temperature program was: initial temperature of 80ºC (hold 2 min), rising at the rate of 25ºC/min to 170ºC, at the rate of 3ºC/min to 200ºC and at the rate of 8ºC/min to 280ºC (hold time 10 min). The total run time was 35.60 minutes. The carrier gas (helium) flow rate was 34.7 ml/min, in constant flow mode. Dwell time was adjusted so that the number of cycles per second was 1.4 throughout the chromatographic

run, providing a sufficient number of chromatographic points. Identification of the studied analyte was done by comparing mass spectra and retention times of the samples barley malt with working standard solutions of methidathion. The identification was confirmed by comparing the relative abundances of the four ions (one quantifier and three qualifiers) of the experimental standards against well-known relative abundances of the US National Institute of Standards and Technology (NIST) library reference spectra. The mass spectrometer was calibrated with perfuorotributylamine (PFTBA). An Agilent Chem Station was used for instrument control, data acquisition and evaluation.

Validation of the analytical method

Fortified samples were prepared by spiking 10 g of barley malt, previously homogenized with appropriate volumes of working standard solutions of methidathion ranging from 0.05 to 1 µg/ml. Limit of detection (LOD) for the analyzed methidathion was estimated from fortified samples. Signal-to-noise (S/N) ratios reported by the instrument software were used to calculate the analyte concentration that yielded a signal-to-noise ratio of 3 for the quantitation of the monitored ion. The limit quantification (LOQ) was calculated from LOD.

RESULTS AND DISCUSSION

Under the selected conditions, the linearity of the calibration curve was evaluated in a concentration range between 0.001 and 0.1 µg/ml using five calibration solutions prepared in methanol. Calculations were based on peak areas. Linear regression was used for quantification and calibration curve did not pass through the origin. The fit of the calibration function was plotted but it was also numerically tested by linearity coefficients, calculated on the basis of curve slope and its deviation ($r = (1-S_b/b) \times 100$). Linearity of methidathion was evidently attained in the whole investigated range. The retention time of the peak and the four ions (145, 85, 93 and 125 m/z) were used for positive identification of methidathion in the samples (Mastovska and Lehotay, 2004). The retention time of methidathion was 22.19 minute. The repeatability of the retention times and peak areas were checked by injecting the standard methidathion solution five times. The relative standard deviations (*RSD)* of the retention times and peak areas were found to be less than 0.1%. The analytical parameters for the developed method of methidathion determination in barley malt samples are presented in Table 1.

Table 1. Analytical parameters for GC-MS detemination of methidathion

Parameter	Concentration interval	Slope[a]	Intercept[a]	Correlation coefficient[a]	LOD	LOQ
	µg/ml				mg/kg	mg/kg
Methidathion	0.001-0.1	9.88×10^3	3.07×10^2	0.9997	0.002	0.004

[a] *Y = a + bc, where c is concentration in µg/ml and Y is response*

The standard addition method was used for methidathion determination in order to eliminate the matrix effect. Barley malt spiked with the concentration levels of 0.02 and 0.04 mg/kg was used to ensure method accuracy (recovery) and data precision. As can be seen in Table 2, the determined amount of methidathion agreed well with the added amount of methidathion. Methidathion recovery in fortified samples was carried out by comparing the detector responses for each independent sample against those measured for calibration standards, which were injected both before and after each fortified sample.

Table 2. Quantitation ion, identification ion, retention time and average recoveries (%) for representative barley malts fortified with 0.02 and 0.04 mg/kg levels

Parameter	Quantitation ion	Identificationion	RT	0.02	0.04
		(m/z)	(minute)	mg/kg	mg/kg
Methidathion	145	85, 93, 125	22.19	93 ± 1.2	95 ± 2.0

The mean recoveries for these two spiking levels ranged from 91 to 96%, with the associated relative standard deviations (RSDs) ranging from 1 to 3%. The small losses in recovery were probably due to matrix effects and suppression of ionization. The final extracts of barley malt samples made by the proposed SPE method were satisfactorily clean for direct GC-MS analysis. A comparison of SIM chromatograms for blank barley malt, malt fortified with 0.02 mg/kg and malt extract is given in Figure 3.

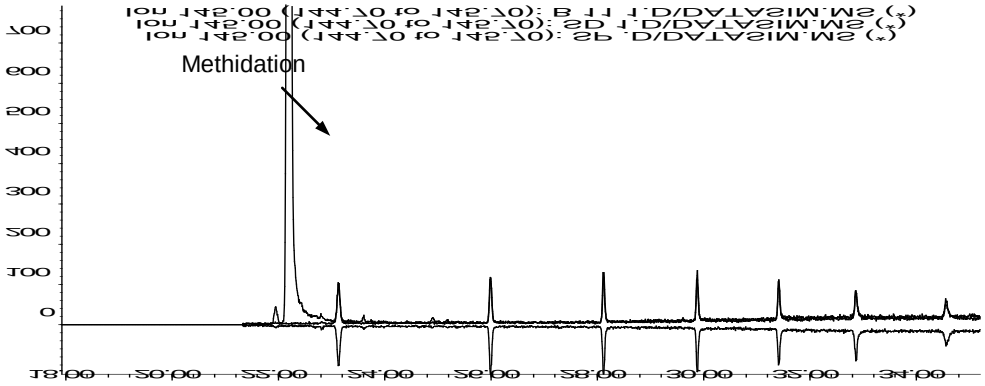

Figure 3. Comparison of SIM chromatograms for blank barley malt extract (lower), fortified barley malt (upper) and barley malt extract (middle)

Besides the regularly shaped and well-defined peaks belonging to the investigated pesticide, the SPE-GC-MS chromatograms (Figure 3) contained a group of peaks which were likely to have originated from the barley malt matrix. There were no significant interfering peaks in the elution region of methidathion. The range of methidathion concentrations in the twenty barley malt samples ranged from 0.004 to 0.018 mg/kg. The obtained positive results could be attributed to the high sensitivity of the developed GC–MS method, as well as to the fact that most cereals grain samples were collected from granaries in which the stored grain had been treated with pesticides in order to control pests.

CONCLUSIONS

This paper describes a SPE-GC–MS method developed for determination of methidathion in difficult matrices such as barley malt. Acetonitrile was used for methidathion extraction from barley malt. Solid-phase extraction in a C_{18} column was used for methidathion isolation and clean-up of the acetonitrile extract. Advantages of this SPE method include simultaneous pre-concentration of methidathion, partial sample clean-up and water removal (the use of drying agents is necessary), applicability to barley malt analysis, and the use of small volumes of solvent per sample (40 ml acetonitrile and 20 ml mixture of acetonitrile and toluene). The additional clean-up on coupled ENVI Carb and aminopropyl columns significantly influences the recovery of methidathion and improves chromatographic performance by minimizing matrix effects. Using MSD, quantification (through selective ion monitoring) and confirmation are achieved simultaneously. The recoveries achieved by the method ranged between 91 and 96% for methidathion, with the associated relative standard deviations (RSDs) ranging between 1 and 3%. Calibration dependences and corresponding regression coefficient values showed that linearity was successfully achieved in the SPE-GC-MS measuring procedure and within the concentration range studied. The corresponding range of methidathion concentrations in the twenty analyzed samples of barley malt was from 0.004 to 0.018 mg/kg. These results were below the permissible levels (MACs) set by Serbian legislation (Official Gazette of RS, 28/11) and the related EU regulations (Council Directive 90/642/EEC, 1990) for methidathion in barley grain (0.02 mg/kg), while these legislations do not regulate methidathion MAC for barley malt.

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EXAMINATION OF RHEOLOGICAL PROPERTIES OF WHEAT SAMPLES BY NEAR INFRARED SPECTROSCOPY

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ABSTRACT: Owing to its several advantages near infrared (NIR) spectroscopy is a widespread analytical method being used in agriculture and food industry as well. It is suitable for identification, qualification and quantitative analysis too. In wheat quality control rheological characteristics of dough made from wheat flour are as important as physical and chemical properties too. Rheological properties like farinograph, alveograph, extensograph parameters are influenced by many factors. For these parameters developing NIR calibration equations with good accuracy is complex problem, prediction of these parameters is more difficult than the chemical composition. In this study we examined wheat samples from different growing area by alveograph and spectral data were collected by FOSS Infratec 1241 instrument. Our first objective was whether it is any effect of the growing area on NIR spectra. Principal Component Analysis (PCA) was developed to examine this effect. We have found that samples from same growing area are close to each other, but there is not any separation in the whole population. According to these PCA results no need to separate the population, we could develop calibration equations on the whole sample set. After qualitative analysis modified partial least squares analyses on NIR spectra were developed for two alveograph parameter (P/L és W) to get calibration equations.
Key words: *wheat flour rheology, NIR, PCA, mPLS*

INTRODUCTION

The study of rheological properties of wheat flour dough plays an important role in the determination of bread-making quality of wheat and has such importance as physical or chemical properties. In last decades, influenced by requirements of the countries and processors a growing use of alveograph and extensograph may be observed. These tests require more sample, time, work and cost, therefore flour quality control needs rapid, reliable tools.

The near infrared spectroscopy such a non-invasive technique, which – owes to its several advantages – widely used in the different industries. The short measuring time of the method makes it possible to get reliable results quickly, in few seconds. This method is applied for not only quantitative analysis than identification or qualification. In agriculture, food industry or other industries there is a higher importance of such techniques which are able to give information with a proper accuracy in short time. If it is identification of raw materials, or prediction of a concrete parameter, near infrared spectroscopy is a good tool for. As this technique is indirect we need a proper sample population for identification, qualification or quantification. This sample group have to be numerous, and representative for the given property (e.g. origin, producer) or parameter (e.g. moisture or protein content). Before developing a calibration model, it is necessary to collect spectra of these samples and to examine the spectra. We can examine the spectra with several chemometric tools, but the Principal Component Analysis is the most common.

Spectra are holders of complex information from chemical and physical aspects too. Composition of the proper sample set for developing calibrations and examination of the spectra is unavoidable. It is necessary even if the laboratory tests show that the sample set

is representative, because there would such variability in the spectra which can cause problem later. Mathematical treatments (e.g. derivation, multiplicative scatter correction, standard normal variate and detrend) are applied to eliminate the differences caused by the physical properties (e.g particle size distribution) of the samples and for example the derivation can solve the problem of separating the overlapping of the peaks (Osborne, 2006). Quality parameters were examined by near-infrared spectroscopy in several studies. In case of moisture and protein content calibration models were made with high accuracy ($0.99<R^2$) (Miralbes, 2003). Miralbes also found that not only these parameters but gluten content is also predictable with almost the same accuracy (R^2=0.95-0.97). Rheological parameters prediction is also in focus. According to Jirsa and co-workers (2008) alveograph P and W value can be predict with $0.8<R^2$. Hruskova and co-workers (2001, 2003) have some studies on wheat rheological properties (alveograph, extensograph) prediction with NIR spectroscopy. They have got different R^2 values for the parameters, but even for extensograph characteristics their accuracy is high ($0.7<R^2$). In the work of Miralbes from 2004 he found that even for the farinograph parameters and for the alveograph parameters the calibrations have high R^2 values, higher than 0.7. Rheological parameters influenced by many factor. The species, the protein content, the gluten composition and other parameters affected the rheological values. Prediction of rheological parameters by near-infrared spectroscopy is a complex problem. The rheological parameters are not linked to concrete bands and wavelengths such as protein or moisture content, because of the affecting factors mentioned above. Therefore the prediction of rheological parameters has lower accuracy than for example for moisture content.
The aim of this study was to examine wheat samples from different origin (grown in different regions of Hungary). We would like to know whether the origin have effects on the spectra, the samples belonging to the different regions show separated groups and the whole sample set could be applied for developing calibration equations on alveograph parameters.

MATERIALS AND METHODS

We examined 207 wheat samples, which were harvested in different regions of Hungary (11 places). Some of these places are arable lands, and one is experimental field.
Infratec 1241 Grain Analyzer (Foss Analytical AB, Sweden) instrument was used for collecting spectra in the range of 850-1050nm from wheat grains. WinISI II v1.50 (Infrasoft International LLC., USA) software was used for the examination of spectra with PCA and for developing calibration equations with mPLS (modified partial least squares regression) on alveograph P/L and W value. For spectra collection we scanned the samples twice.
We used modified partial least squares method with cross validation to examine the relations between the spectra and reference values and develop calibration models. First we did not use any math treatment, but later we applied SNV (standard normal variate) and first derivate (1, 4, 4, 1).
Rheological properties were measured by Chopin alveograph (Tripette & Renaud, Villeneuve La Garevne, France) according to the approved method A.A.C.C. Standard No. 54-30A (A.A.C.C. International, 2000). The recorded alveograph parameters included the biaxial extensibility (L), the maximum overpressure (P), the swelling index (G), the deformation energy (W), and the configuration ratio (P/L).

RESULTS AND DISCUSSION

After collecting spectra we examined them with PCA whether there is any separation between the samples or whether there is any outlier ones. PCA results shows, that the first three principal components are responsible for more than 99% of the total variance in the spectra (Table 1).

Table 1.Individual and cumulative values for the first three principal components

		Individual	Cumulative
PC1	PC1	98.44	98.44
PC2	PC1+PC2	1.48	99.92
PC3	PC1+PC2+PC3	0.05	99.97

Results gave two samples as outliers, and we eliminated them from the sample set.
In graphical interpretation of the results when we signed the samples coming from the same area, we did not see any separation. Samples belonging to the same growing area are close to each other, but these groups are not separated from each other, there are overlaps. This means that we could use the whole sample set for developing calibration models without making different groups and separate models for each growing area.

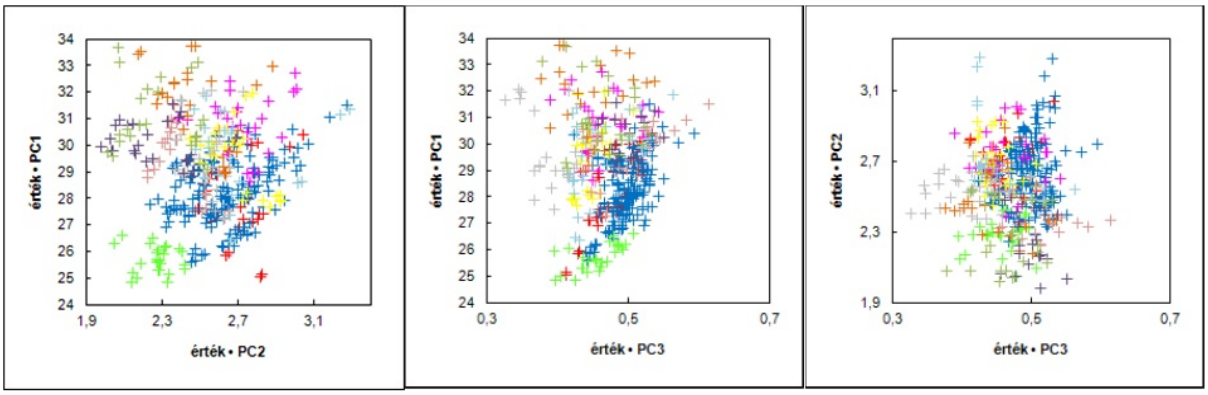

Figure 1. Crop site based coloured presentation of samples for the sample population

The number of the samples was enough high, and according to the laboratory tests we can say that our sample set is representative for the alveograph parameters, these values (P/L, W) are in a wide range (Table 2).

Table 2. Summary of the reference parameters

	P/L	W (10^{-4}J)
Minimum	0,20	43
Maximum	3,31	446
Mean	1,10	244
SD	0,58	84
n	207	207

Table 3. Statistical parameters of NIR calibrations

	n	n_{cal}[1]	Terms	Mean	SD	SECV[2]	R^2	SEP[3]	RPD[4]
P/L	410	394	11	1.07	0.50	0.30	0.64	0.39	1.28
W	410	392	9	246	80.65	39.42	0.75	51.24	1.57

[1] *n_{cal} - number of samples selected for calibration*
[2] *SECV - standard error of cross-validation*
[3] *SEP - standard error of prediction*
[4] *RPD - SD/SEP (Williams, 1993)*

The summary of statistical parameters of the NIR calibrations is showed in Table 3. According to R^2 values alone, both parameters could be predicted with good accuracy, because R^2 values are 0.64 and 0.75 for P/L and W, respectively (Figure 2, Figure 3). For accurate evaluation of the model, we have to see other parameters as well. SEP (standard error of prediction) and RPD (ratio of standard deviation to SEP) must be under consideration. SEP values show bad performance, because in case of P/L value is 36.7% to

mean, and in case of W value is 20.8%. The error for standard method is about 5% and SEP would be close to this value and higher because of the NIR spectroscopy is an indirect method. The SEP values should be minimised for better calibrations. According to Williams (1993) RPD should be as high as possible. If RPD is higher than 10, the calibration is excellent; between 5 and 10 is adequate for quality control; 2.5 and over are satisfactory. We calculated the RPD values for both models, and we have got low RPD values ($RPD_{P/L}$= 1.28, RPD_W=1.57). These RPD values mean that in spite of good R^2 values, the models have not enough accuracy. Other authors gave lower SEP values and higher RPD values, therefore calibrations with good accuracy.

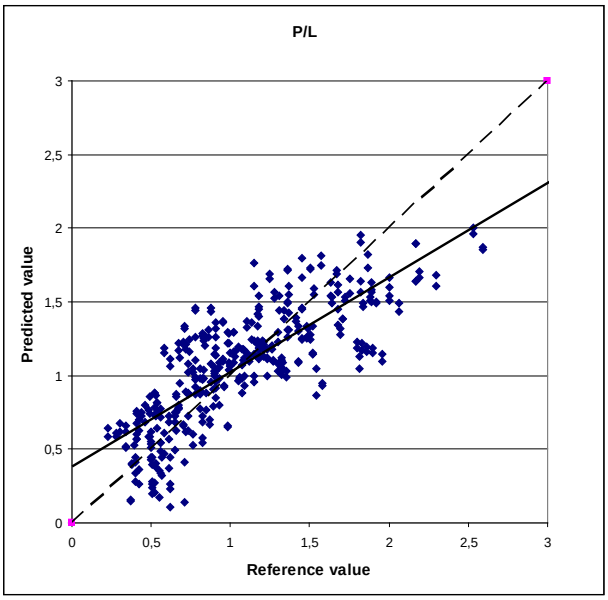

Figure 2. Comparison of P/L determined by prediction model and by reference method

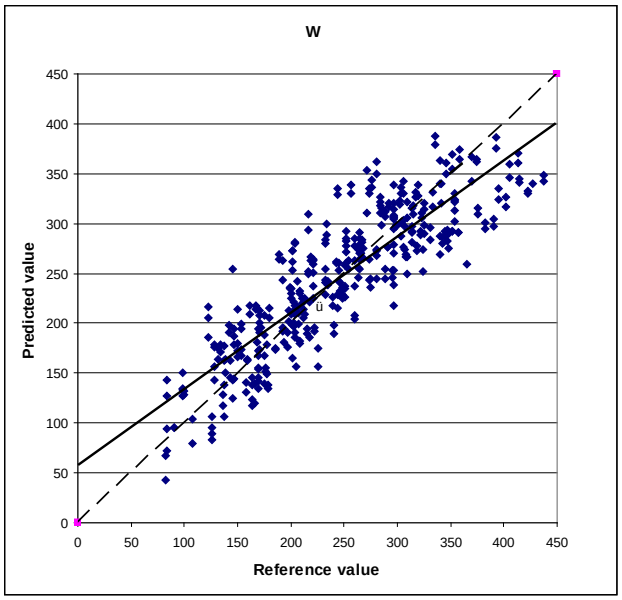

Figure 3. Comparison of W determined by prediction model and by reference method

In case of math treatment of SNV and first derivate the results can be seen in Table 4.

Table 4.Statistical parameters of NIR calibrations with SNV and first derivate

	n	n_{cal}	Terms	Mean	SD	SECV	R^2	SEP	RPD
P/L	410	393	12	1.06	0.51	0.29	0.70	0.38	1.34
W	410	391	9	245	80.33	38.67	0.76	50.27	1.60

[1]*n_{cal} - number of samples selected for calibration*
[2]*SECV - standard error of cross-validation*
[3]*SEP - standard error of prediction*
[4]*RPD - SD/SEP (Williams, 1993)*

Use of SNV and first derivate gave better calibration models. R^2 values are higher: 0.70 and 0.76 for P/L and W, respectively. If we see the SEP and RPD values, these shows better performance than earlier, but the SEP value is still high and RPD is low for good prediction.
Compare to Miralbes (2004) results, he found $SEP_{P/L}$=0.04 and SEP_W=21.5, our results higher, and also for R^2 (0.70 and 0.92, P/L and W, respectively), in case of P/L we have got the same, but for W is lower. RPD values also higher for him (5.7 and 4). Our R^2 value for W (0.76) is higher than for Jirsa and co-workers R^2=0.535 and 0.636 (for two sample sets), but they got lower SEP values.
In our case, the sample set is numerous and representative, but the calibration models would be more accurate. We need more detailed examination of the spectra to get better accuracy.

CONCLUSIONS

In our study we examined wheat samples with NIR spectroscopy and with alveograph rheological test. After collecting spectra PCA was carried out to get information about differences in sample set. PCA showed that there is no difference between the samples from the different growing areas, therefore we can use the whole sample set to develop calibration equations on alveograph rheological parameters (P/L, W). MPLS method was used for calibration, and we have got good R^2 values for both parameters, but evaluation the models by RPD and SEP, these calibrations are not able to predict these two alveograph parameters with good accuracy.

ACKNOWLEDGEMENTS

This work was supported by the OTKA 68706 project: "Az ökotermesztés hatása a biológiai aktivitással rendelkező másodlagos metabolitok képződésére" (Effect of organic farming on the formation of biologically active secondary metabolites).

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FACTORS THAT AFFECT THE QUALITY OF MEAT

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ABSTRACT: The quality of meat depends on the composition and properties of both materials used and conditions of processing. The quality of derived meat can vary under the influence of environmental factors, conditions of cultivation and transportation, ante livestock slaughter conditions and initial processing, refrigeration storage options as well.Therefore a united approach comprising consistent standards robust controls is required to ensure consumers' health and to maintain consumers' confidence and satisfaction. Chemical analysis along the whole food chain downstream (tracking) from primary production to the consumer and upstream (tracing) from the consumer to primary production is an important prerequisite to ensure food safety and quality. In this frame the focus of the following paper is on the "chemical safety of meat and meat products". It should be taken into account inorganic and organic residues and contaminants, the use of nitrite in meat products, the incidence of veterinary drugs, as well as a Failure Mode and Effect Analysis (FMEA) system assessing (prioritizing) vulnerable food chain steps to decrease or eliminate vulnerability.
Key words*: beef, quality of meat, chemical safety of meat, breed, carcass quality*,

INTRODUCTION

The objective of this article is to provide with basic information on different factors that influence meat quality as guidelines for the industry. We have simply defined different areas and factors that influence meat quality. Much of this information is basic knowledge.
This paper is concerned with quality in a broad commercial sense and reviews some of the quality attributes which are important in market place. There are many definitions of quality, but one of them takes into account the need to match products quality to the demands of the market place: "Quality is the composite of those characteristics that differentiate individual units of a product and which have significance in determining the degree of acceptability of that unit to the user". Of course, price is a part of the customer's appraisal of what is offered in the market place and sometimes it is the all important feature. In other market places demand massively exceeds supply and distribution is the main marketing concern. If the product has no genuine appeal, then price will certainly be the key feature.
Meat quality is an essential trait in meat-producing animals, especially pigs. With progress in breed selection and nutritional or non-nutritional manipulations, porcine growth performance has improved significantly and the amount of meat production has correspondingly ncreased. Conversely, however, pig meat quality has decreased. How to improve meat quality while maintaining or increasing the amount of pig meat production is therefore an ongoing challenge.

MATERIAL AND METHODS

Study of animals on the farm were performed during 2010-2011. A number of 2,000 animals were tested and investigated their feeding rations and living conditions. After that such products of slaughter as meat, animal blood, liver, intestine was investigated.

Analyses

Forage quality and balance. Sanitary condition of the animals was determined by organoleptic and visual methods.

RESULTS AND DISCUSSION

The quality of the finished product depends on the composition and properties of materials used, conditions of processing. The quality of derived meat can vary under the influence of environmental factors, conditions of cultivation and transportation, ante livestock slaughter conditions and initial processing, refrigeration storage options (Figure 1).

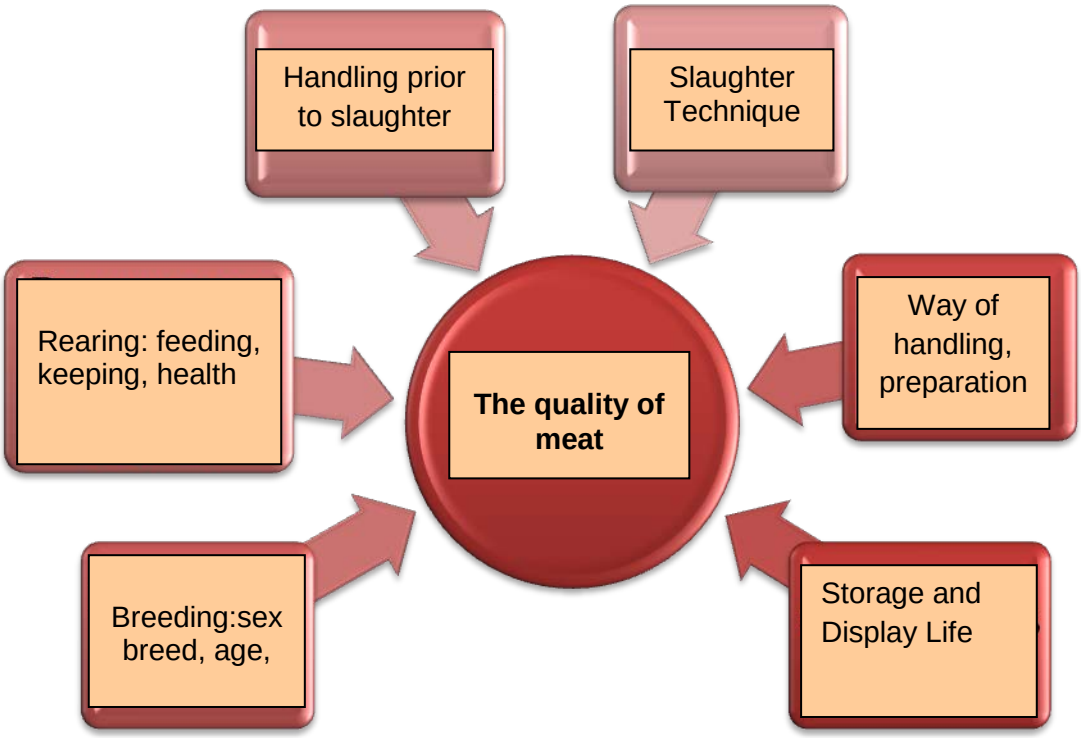

Figure 1. Factors that affect the quality of meat.

Effect of breeding phases on the meat quality

Animals of different species have significant differences in body weight, and quality of meat. Meat breeds of cattle have well-developed muscle and adipose tissue is considered to be juicy, tender and delicious. Meat derived from milk, meat and dairy breeds, characterized with an increased amount of bone and connective tissue have fewer intramuscular fat and inferior organoleptic characteristics.

Animals sex affects the quality and quantity of meat derived. Castration of animals make impact on the growth rate and efficiency of digestion of food animals, and the output of meat. Sex differences in the meat of young animals is less pronounced. With age the meat in males compared with females increases moisture content while reducing the content of protein and fat. At the same time in the flesh of bulls increasing proportion of connective tissue appears dark. Castrated animals are developed more slowly, but meat that is obtained from them has a distinctive picture "marbleness."

With age, the animal meat is coarser due to thickening of the muscle fibers, increasing the proportion of elastin fibers in connective tissue and strengthening of the collagen fibers. The degree of hydrothermal dissolution of collagen from the meat of animals aged 12 months is 40.6% at the age of 8 to 10 years is 21.5%. Changing the chemical composition of meat: fat content increases, the amount of water decreases. At the age of 12 to 18 months, the ratio of the main components of cattle meat is the best value for money. In pigs the best quality characteristics are formed to 8 months.

To ensure the identity of qualitative raw materials used in sausage production, cattle at slaughters are divided into groups: animals older than 3 years (adult cattle meat) and at the age of 3 months to 3 years (young meat animals).The older is an animal the tougher is the

meat. For this reason main stream livestock industries have established grades and/or names to account for the age of the animal. They have also established means for determining the age of the animal. Depending on the specie it may be by teeth development, degree of bone ossification or similar. Provided the animal meets the basic health criteria all meat is fit for human consumption, with different ages having different uses, different markets and therefore different price structures.

The influence of the feeding ration.

The feeding ration influences the quality characteristics of meat. The correlation of the coarse fodder and the concentrates in the ration, the degree of the macro- and micro elements balance, the high energy value define the forming of the high taste qualities of meat and its technological properties. The insufficiency of feeding rations displays the decrease of the category of animal's fatness, the rise water maintenance in meat, the shrinkage of muscle fibers, and the rise of toughness.

The change in the structure of the feeding ration allows getting meat with necessary characteristics. The colour of the muscle tissue of pigs that got the ration with corn and barley is much more intensive than while feeding only with corn.

The influence of the cattle's maintenance conditions.

Conditions of cattle's maintenance that include the way of animal's growing, climatic and weather conditions also affect on the quality of meat. Pork that comes from industrial complexes contains a big amount of muscle tissue and corresponds to meat fatness. In a result of violation of the diet and also because of the increased susceptibility of animals to stress during the mass maintenance, raw materials may have low quality rates that are expressed by specific odour and tastes (fish, oil) by introducing the fish flour and protein additives of microbiological origin into the fodder.

Handling prior to slaughter.

During the time between leaving the farm and slaughter, animals are subjected to removal from their home environment, loading and unloading from vehicles, transport, and holding in unfamiliar surroundings. They may be exposed to stressors such as noise, strange odors, deprivation of food and water, vibration and changes of velocity, extremes of temperature, breakdown of social groupings, close confinement and often overcrowding. These stressors often elicit behavioral and physiological responses, some of which can, if extreme, contribute to a reduction in carcass and meat quality. Herein is the link between welfare and quality.

Table 1.Factors that affect the quality of meat on stage ante animals

№	Factor	Provided that the quality of meat
1.	Diet feeding in the final period of growth and the transport	adherence to diet
2.	Loading and unloading	Reducing the duration of operations, stress effects eliminate
3.	impact the environment	Control of ambient temperature, the relative humidity, air, light
4.	healthy of animals	Do not allow to transport the sick and debilitated animals
5.	Method livestock during transport	Individual livestock by type, sex and age
6.	Handling prior to slaughter	Compliance with conditions, eliminating stress and impacts

Reduction the likelihood of stress in animals allows compliance:

•Support sustainable animal feeding diet in the last 7 to 10 days of their stay at the industrial complex, sometimes recommended to provide livestock feed in excess to increase the supply of glycogen in the muscles, for 10 to 12 hours before the loading animals should be stopped feeding;

•Reducing the period of loading and unloading animals on vehicles, careful treatment of animals during this period to reduce the physical and psychological stress;

•Avoid the impact on animals External factors: abrupt fluctuations in temperature, drop in atmospheric pressure, changes in relative humidity, bright light, ambient temperature during transport must not go beyond -5 to 20 ° C to eliminate drafts, the effect of direct sunlight; monitor the circulation of air in the summer of pigs better transport at night or morning;
•Do not allow the state of physical fatigue in animals during transport;
•Do not allow to transport physically frail and sick animals;
•The duration and speed of transport should be minimal;
•Vehicles must be properly fitted, equipped with ventilation, partitions to separate the animals, feeding troughs;
•Compliance with veterinary and sanitary requirements and zoohihiyenichnyh Pets;

CONCLUSIONS

Main goal of selection and other measures in animal breeding is to increase the quantity of major meat sections in carcass sides but with preserved quality of meat, i.e. that adequate hygiene-toxicological, technological, chemical and sensory traits are maintained. Quality of meat carcasses has effect on competitiveness of production and market demands. Research was carried out in order to investigate the effect of sire breed and sex of progeny on quality of carcass and meat. Based on the research it can be concluded that investigated factors can have significant effect on certain traits of carcass quality, as well as on technological and biological traits of meat For investigated chemical traits of meat no effect of investigated factors was established.

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POST- HARVEST FUNGI PRODUCTION IN DIFFERENT STORAGE CONDITIONS OF THE CEREALS

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ABSTRACT: Crops are exposed to fungal contamination both on the field before harvest, and especially during storage for longer periods of time in improper conditions. Concerning the origin of fungi contamination the following sources exist: contamination from the warehouse or silo dispersion, the predominant fungi is Aspergilius and Penicillium, contamination remaining from the field before harvest with: *Clamidosporium, Fusarium, Mucor, Rizopus* and advanced alteration, which associates more rare types in remanent contamination.
In this paper, the influence of storage conditions on the fungi growth and mycotoxins production in cereals was studied.
Cereal samples (wheat, maize, barley and oats) collected from warehouse, individual silos and storage cells from the west side of Romania were analysed. The cereals were sampled from private producers, who reported the information regarding storage conditions. The storage period ranged between 6 months and over 1 year. The main measures of conditioning applied to samples before storage were related to: drying, pest removal, aeration and cleaning. Also, the main parameters (temperature, humidity, ventilation) during the storage were monitored in order to preventthe toxins production.
The method used for fungal species with mycotoxigen potential identification was microbiological assay.
The experimental results indicated that the fungus species isolated and identified were *Aspergillus (flavus and terreus, Fusarium roseum, Penicillium sp., Chaetomium sp. and Cladosporium sp*. The most frequent *Fusarium fungal* species in cereal samples were identified F. culmorum, DON mycotoxin producers, *F. subglutinans*, fumonisin producers and *F. acuminatum*, non mycotoxin producer. *Fusarium species* was identified in 45 % maize samples, 25% wheat samples, 100% triticale and barley samples and 0% in oats samples.
In order to avoid the production of mycotoxins after harvest, it is advisable to dry the grain and bring to optimum moisture retention, less than 14%. Also, post-harvest contamination can be avoided by monitoring and keeping optimal grain storage conditions.
Key words: *fungi, storage, cereals, harvest.*

INTRODUCTION

Grain entering store carries a wide range of microorganisms including bacteria, yeasts and filamentous fungi, the population structure being dependent on field climatic conditions and harvesting processes (Lacey and Magan, 1991)
Crops are exposed to fungal contamination both on the field before harvest, and especially during storage for longer periods of time in improper conditions, which have favourable environments for mold development. Concerning the origin of fungi contamination the following sources exist : contamination from the warehouse or silo dispersion, the predominant fungi is *Aspergilius and Penicillium*, contamination remaining from the field before harvest with: *Clamidosporium, Fusarium, Mucor, Rizopus* and advanced alteration, which associates more rare types in remanent contamination with *Sordaria, Papulaspora and Fusarium*, including in this group also unidentified strains and advanced alteration, that associates more rare types in remanent contamination (Alexa, 2008).
The main factors and conditions for fungal development and production of toxins are: *genetic ability of fungi* (it was found that among the many species of fungi only a few grow on certain

agricultural products, and of these only some produce mycotoxins), *substrate* (it was found that the best substrate for the accumulation of toxins is oil seeds and cracked grain), *moisture of the substrate and the environment*. The grain humidity of 13-15% prevail *Aspergillus glaucus*, and to over 15% grow *Aspergillus flavus, A. ochraceus, A. versicolor, temperature* influences both the development of molds and mycotoxin production, *compositions of the atmosphere* influence in a smaller degree the formation of mycotoxins (Magan, 2003; Dumitrache, 1997). Lund and Frisvad (2003) showed that P. Verrucosum contaminated grain during the harvesting process and during drying and storage.
Control of mycotoxins is the need of the day, since their occurrence in foods and feeds is continuously posing threats to both health and economics all over the world (Bazollou, 2009). Prevention of fungal infection during plant growth, harvest, storage and distribution and the measures that must be taken for decontamination is an actuality issue for European Commision [9]. The pre-harvest selection of hybrids, time of planting, plant density and insect control have all be found to have an impact on contamination of maize with mycotoxins pre-harvest and during drying and storage (Magan, Aldred, 2007; Magan et al., 2010).
Storage in adequate conditions (moisture, temperature and insect control) and the addition of antifungal agents may diminish fungal growth but can not detoxify contaminated samples. The previous studies have shown that it is essential that grain is dried to lower moisture contents as quickly as possible regardless of the drying system employed (Cairns Fuller et al., 2005). The most important factors governing the life cycle of micro-organisms are water availability, temperature and their interaction with the nutrient status of the food matrix. The limits for growth and OTA production are approx. 17–18% moisture content (ca. 0.80–0.83aw) (Magan Naresh, Alfred David, 2005*).*
General rules to prevent mycotoxins contamination in the warehouse involve: regular and accurate moisture measurement determinations for cereals, ensuring adequate transport conditions of grain, ensuring appropriate storage conditions at all stages in terms of moisture and temperature control, the general maintenance and effective hygiene of storage facilities for prevention of pests and water ingress, ability to efficiently identify and reject material below specified standards in terms of fungal contamination (Molinié and Pfohl-Leszkowicz, 2003; Alexa et al., 2012).
This paper has studied the influence of deposition conditions on the fungi production of cereals storage in different counties of Romania.

MATERIAL AND METHODS

The method used for fungal species with mycotoxigen potential identification was microbiological assay.
Determining the degree of colonization of seeds with *Fusarium sp.* was achieved by the technique of direct isolation on specific culture media. The medium used was CZID (Czapek iprodione dichloram agar) (Pitt and Hocking Ailsa, 2009). First, the seeds were disinfected with on the surfaces by immersing seeds in chlorine solution 0.4%, for 2 minutes then drain the chlorine. After that the samples were washed thoroughly in sterile water for 2 minutes; driedand plated on surface media in Petri plates. We used an average sample of which 10 seeds were filed on the medium. We worked in three replicates. After seven days, we identified the cultures as genus *Fusarium.* To distinguish species of *Fusarium* we have used the observations about the colour and morphology of colonies, the size and shape of the macroconidia, the presences or absences of the microconidia and the type of phialide on witch microconidia are produced. (Sumalan R., et al., 2011).
The technological conditions during the storage were esthablished on the base of questionnaires that have been completed by farmers in order to determine the causes that lead to fungal contamination of crops in west Romania counties (Timis, Arad, Bihor and Satu Mare).

RESULTS AND DISCUSSION

Cereal samples (wheat, maize, barley and oats) collected from warehouse, individual silos and storage cells from the west side of Romania were analysed. The cereals were sampled from private producers, who reported the information regarding storage conditions. The storage period ranged between 6 months and over 1 year. The main measures of conditioning applied to samples before storage were related to: drying, pest removal, aeration and cleaning.

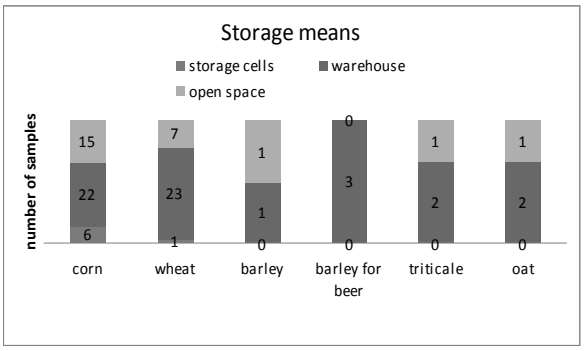

Figure 1.Type of cereals

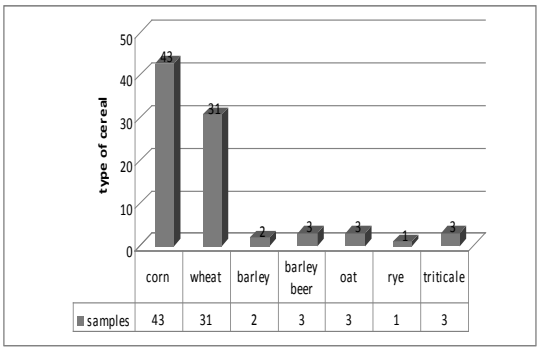

Figure 2. Storage means

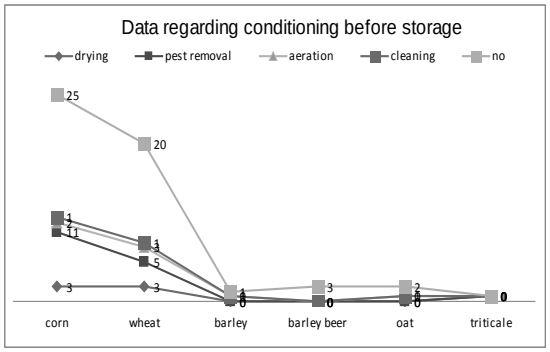

Figure 3. Storage period

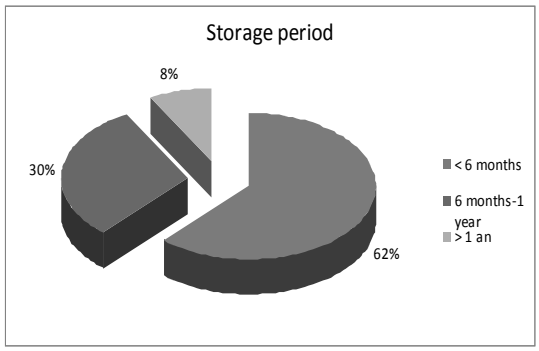

Figure 4. Conditioning measures

From all 86 samples collected from private producers, 43 were corn samples, 31 were wheat samples, 2 barley, 3 oat, 3 barley for beer, 3 triticosecale and 1 rye (figure 1). Regarding storage means, from all 43 corn samples, 22 pursued from warehouses, 15 from open spaces and 6 from cells (figure 2). From all 31 wheat samples, 23 were from warehouses, 7 were from open spaces and 1 from cell. Barley, barley for beer, triticosecale and oat were pursued from open spaces and warehouses (figure 2).
The storage period of sampled cereals ranged between 6 months and over 1 year (figure 3). Regarding conditioning measures, most of the samples were not subjected to any of the conditioning techniques, so from all 43 corn samples, 25 were not subjected to any conditioning measure, 11 samples were subjected to pest removal, 3 samples were subjected to cleaning, 2 aeration and 1 drying (figure 3). Barley, barley for beer, oat and triticosecale were not subjected to any of the conditioning measures (figure 3).
Also, main storage parameters were monitored (temperature, humidity, ventilation) in order to prevent the toxins production.
From all 43 corn samples 8 were subjected to temperature control, 8 to moisture control, 8 to ventilation, 8 to infestation control and 3 to other measures. Regarding wheat samples, out of 31 samples, 6 were subjected to temperature control, 2 to moisture control, 8 to ventilation, 8 to infestation control, 3 to pest control and 4 to other measurements (*figure 5*).

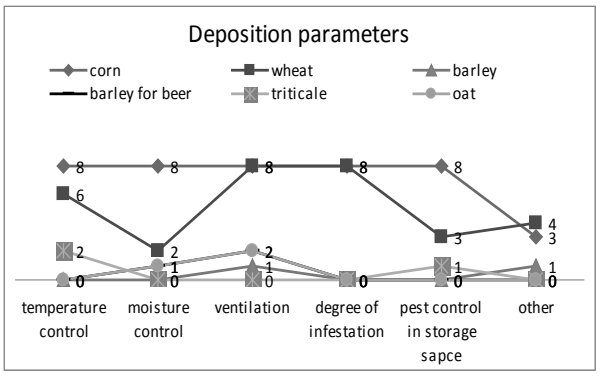

Figure 5. Deposition parameters

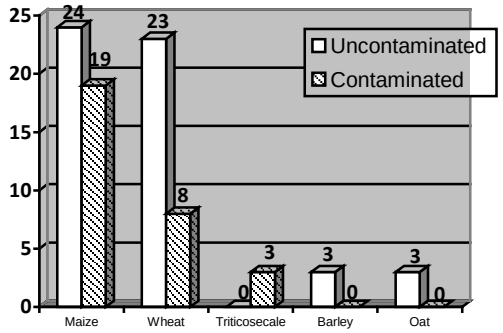

Figure 6.The number of contaminated and uncontaminated samples with Fusarium

The experimental results indicated that the fungus species isolated and identified were *Aspergillus (flavus and terreus, Fusarium roseum, Penicillium sp., Chaetomium sp. and Cladosporium sp.* The most frequent Fusarium fungal species in cereal samples were identified: *F. culmorum*, DON mycotoxin producer, *F subglutinans*, fumonisin producers and *F. acuminatum*, non mycotoxin producer. *Fusarium* species was identified in 45% maize samples, 25% wheat samples, 100% triticale and barley samples and 0% in oats samples (figure 6).

CONCLUSIONS

Storage conditions affect fungal charge of grain. In the grain samples analyzed from counties in western Romania, the predominant fungi were of the species *Fusarium,* especially *F. culmorum*, *F subglutinans* and *F. acuminatum,* fungal charge being due to the absence or failure of optimal storage conditions.An important role in fungal contamination is due to temperature and humidity, ventilation, infestation degree and pest control.

ACKNOWLEDGEMENTS

This paper was achieved from research project funds: *Systems to reduce mycotoxins contamination of cereals and medicinal plants in order to preservation native species and traditional products in Romania - Serbia - Croatia area,* financed by SEE-ERA NET PLUS Program, Contract reference ERA_139/01, 2010-2012.

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ANTIMICROBIAL ACTIVITY OF POLYSACCHARIDES EXTRACTED FROM MEDICINAL MUSHROOMS ON *Campylobacter jejuni* AND *Staphylococcus aureus*

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ABSTRACT: Modern consumers demand for fresh and minimally treated food products is dictating a trend of reducing the use of man-made chemical preservatives, although this could increase health risk, caused by foodborne pathogens and spoilage. Therefore the interest in finding new natural antimicrobials and antioxidants is increasing. We investigated antimicrobial activity of hot water and hot alkali extracted polysaccharides from selected medicinal mushrooms, which are known for their broad spectrum of health promoting activities. The most effective against *Staphylococcus aureus* and *Campylobacter jejuni* were crude hot water extracts of *Phellinus linteus* and *Ganoderma lucidum*, composed of a mixture of polysaccharides and phenolic compounds and partially purified extract of *G. lucidum*. MIC (minimal inhibitory concentration) of *P. linteus* and *G. lucidum* crude extracts were 5 mg/ml and >10 mg/ml on *S. aureus*, and 10 mg/ml on *C. jejuni*, respectively. These extracts showed considerable bacteriostatic effect against gram-negative *C. jejuni* already in concentration of 0.25 MIC. Lower antimicrobial effect was observed on *S. aureus*.
Key words: *antimicrobial activity, mushroom polysaccharide extracts, anti-Campylobacter activity*

INTRODUCTION

Many medicinal mushrooms have been proven to contain biologically active components, expressing different medicinal functions including antitumor, anti-inflammatory, immunomodulating, antioxidant, radical scavenging, cardiovascular, antiviral, antibacterial, antiparasitic, antifungal, detoxificative, hepatoprotective, and antidiabetic effects. Medicinal mushrooms have an established history of use in traditional oriental therapies. Modern clinical practice in Japan, China, Korea and other Asian countries continues to rely on mushroom-derived preparations. In searching for new therapeutic alternatives, they have become interesting subject of research (Wasser 2010; Guillamón et al., 2010; Moro et al., 2012; Hsu et al., 2008; Li et al., 2011).
Special attention is paid to mushroom polysaccharides. Polysaccharide extracts from *Ganoderma lucidum, Agaricus bisporus, Agaricus brasiliensis, Phellinus linteus*, *Schizophyllum commune* have been shown to have antioxidant activity and are health beneficial nutritional supplements, which could also be used as natural food additives to prevent oxidative deterioration (Kozarski et al., 2011; Turkoglu et al., 2007; Klaus et al., 2011). Consumers demand for fresh and minimally chemically treated food is namely increasing. As good as this may be from nutritional point of view, it does arise considerable food safety and quality issues. Less conservation treatment not only reduces the shelf life of food products, but also compromises enzymatic and microbiological stability of food products. The extracts of edible and medicinal mushrooms have been proved to have antioxidant and antimicrobial activity against Gram-positive (Hur et al., 2004; Karaman et al., 2010) and Gram-negative bacteria (Moradali et al., 2005; Li et al., 2012) as well as antifungal activity (Turkoglu et al., 2007). Therefore, they could potentially be used as bacteriostatic

agents in food. To our knowledge, their antimicrobial activity against *Campylobacter* has not yet been tested.
The aim of this study was to investigate the antimicrobial effect of mushroom extracts. Most of the tested extracts were water-soluble. More lipid-soluble extracts were excluded from further testing. Extracts were obtained by hot water extraction from *Ganoderma lucidum*, *Ganoderma applanatum*, *Trametes versicolor*, *Lentinus edodes*, *Schizophyllum commune*, *Laetiporus sulphureus*, *Agaricus blazei, Agaricus bisporus* and *Phelinus linteus*. For further testing, polysaccharide extracts from *Ganoderma lucidum, Laetiporus sulphureus, Phelinus linteus and Agaricus blazei* were chosen. To evaluate the impact of potential impurities on antimicrobial activity of polysaccharide extracts, we further tested water-soluble extracts of *G. lucidum* and *L. sulphureus*, partially purified with dialysis. Antimicrobial effect was tested against Gram-positive *Staphylococcus aureus,* most often used model for testing antimicrobial activity, and Gram-negative *Campylobacter jejuni.* This microorganism is the cause of human campylobacteriosis, which has recently become the world's leading bacterial food-borne illness and the most frequently reported zoonosis in humans (EFSA, 2012; Smole Možina et al., 2011).

MATERIALS AND METHODS

Bacterial strains, culture media and growth conditions

Bacterial strains of *S. aureus* ŽMJ 72 and *C. jejuni* ATCC 33560 were stored at -80 °C and revitalized on Müeller-Hinton agar (MHA) and Columbia blood agar (CBA), respectively. Both cultures were incubated for 24 hours; *S. aureus* at 37 °C and *C. jejuni* at 42 °C in microaerobical conditions. A loop of each culture was transferred in Müeller-Hinton broth (MHB) and 5 % horse lysed blood was added in case of *Campylobacter*. Incubation took place for 24 hours in previously mentioned conditions. Working concentrations in range of 10^4-5x10^5 CFU/ml were prepared by proper dilution of culture in MHB.

Reagents and media

INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) (Sigma-Aldrich, USA), Cell-TiterGlo™ (Promega, Madison, USA), MHB, CBA and MHA (Oxoid, Hampshire, UK). Milli-Q water obtained from a Milli-Q water purification system (MQ) was used for preparing all of the extracts.

Instruments

Microplate reader (Tecan, Mannedorf, Zurich, Switzerland).

Preparation of polysaccharide extracts

Hot water extract, partially purified polysaccharides and hot alkali extracted polysaccharides were prepared as described before (Klaus et al., 2011). Partially purified extract was obtained by dialysis from the crude hot water extract, while hot alkali extracted polysaccharides were obtained from the filter cake originated from the first step of making hot water extract. To remove residual small molecules as polyphenols, peptides and polysaccharides < 8-10 kD, i.e. to partly purify hot water extract, dialysis was done with ZelluTrans/Roth® 6.0 regenerated cellulose tubular membrane (MWCO: 8.000-10.000) against a large volume of MQ for 24 h at room temperature.

Determination of the MICs by broth microdilution method

Broth microdilutions were carried out as previously described (Klančnik et al., 2010). Viability of *S. aureus* and *C. jejuni* was determined with INT and Cell-TiterGlo™, respectively. Minimal inhibitory concentrations were defined as minimal concentrations at which no viability was detected, based on color change or absence of bioluminescence. In case of detection with Cell-TiterGlo™ there was possible to determine also the minimal concentration at which viability started to decrease.

Determination of kinetics of microbial inhibition

Kinetics of microbial inhibition was determined by macrodilution method in MHB. Bacterial cultures were prepared in concentration of 10^4-$5x10^5$ CFU/ml. Each extract was diluted in 1 ml of bacterial culture to reach the concentration of 2.5 mg/ml. This concentration represented 0.25 MIC in most cases and was selected to see potential bacteriostatic effect of subinhibitory concentrations. Sampling was carried out at start and after 3, 6, 9 and 24 hours.

RESULTS AND DISCUSSION

Majority of the tested extracts had better antimicrobial effect against *C. jejuni* than *S. aureus* (data shown in Table 1). All, except hot alkali extract from *L. sulphureus* had MIC of 10 mg/ml against *C. jejuni*, while all, except crude *P. linteus* and partially purified *G. lucidum* extracts had MIC higher than 10 mg/ml against *S. aureus* (Table 1).

Table 1. Minimal inhibitory concentrations of tested mushroom extracts

Mushroom	**Extract type**	***S. aureus* ŽMJ 72**	***C. jejuni* 33560**	***C. jejuni* 33560**
		MIC (mg/ml) at 37 ºC	Start of viability reduction (mg/ml) at 42 ºC	MIC (mg/ml) at 42 ºC
Agaricus blazei	Crude	>10	2,5	10
Phellinus linteus	Crude	5	2,5	10
Laetiporus sulphureus	Crude	>10	2,5	10
Laetiporus sulphureus	Hot alcali	>10	2,5	>10
Laetiporus sulphureus	Partially purified	>10	2,5	10
Ganoderma lucidum	Crude	>10	2,5	10
Ganoderma lucidum	Hot alcali	>10	2,5	10
Ganoderma lucidum	Partially purified	5	2,5	10

Crude *P. linteus* and partially purified *G. lucidum* extracts showed the best antimicrobial activity in microdilution assay, with MICs of 5 mg/ml. Interestingly, the viability reduction in microdilution assay on *C. jejuni* were observed already at 2.5 mg/ml for all tested extracts. Thus we investigated the effect of this, in most cases 0.25 MIC concentration, on kinetic of growth inhibition. Tests were carried out at optimal growth temperature; 37 ºC for *S. aureus* and 42 ºC for *C. jejuni*. We used the same concentration, 2.5 mg/ml, of all extracts on both bacteria. The focus of our interest was to examine whether the extracts show any bacteriostatic activity also in this subinhibitory concentration and to see the differences in antimicrobial activity among extracts with different composition (e.g. crude extracts, hot alcali and partially purified extracts).

Crude extracts from *P. linteus* and *G. lucidum* showed overall best inhibitory effect against both, *S. aureus* and *C. jejuni*, with slight growth reduction of 0.7 and 0.4 $\log_{10}$CFU/ml on *S. aureus* and 3.3 and 2.0 $\log_{10}$CFU/ml on *C. jejuni*, respectively (results showed in Figure 1 and 2). Hot alkali extract showed the best inhibitory effect in case of *S. aureus*, with growth reduction of 1 $\log_{10}$CFU/ml. The extract from *P. linteus* worked bacteriostatically on *S. aureus* in first three hours after starting the experiment, while other extracts did not display a typical bacteriostatic activity. In case of *C. jejuni*, crude extracts from *P. linteus* and *G. lucidum* as well as partially purified extract from *G. lucidum* showed bacteriostatic effect in first nine hours of experiment. These extracts were able to prolong the lag phase of *C. jejuni* already in concentration of 0.25 MIC.

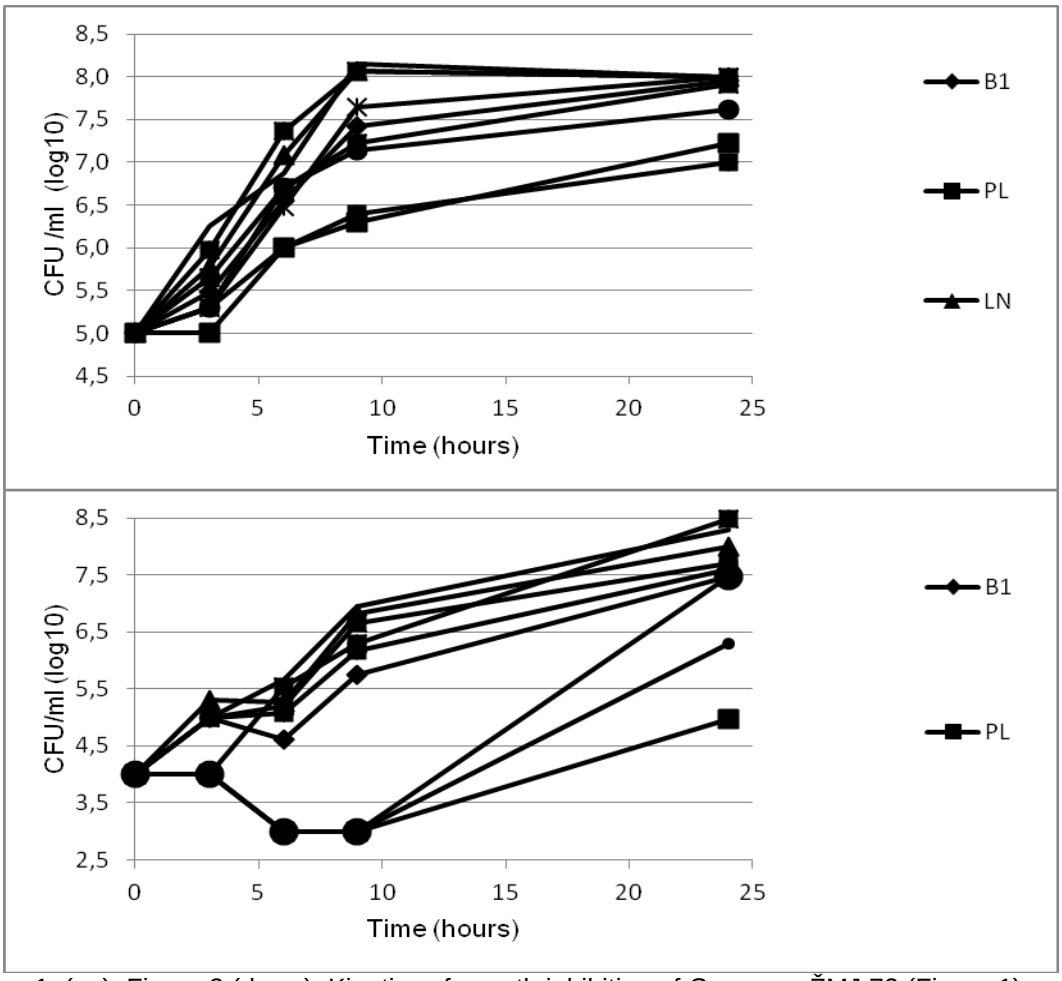

Figure 1. (up), Figure 2 (down). Kinetics of growth inhibition of *S. aureus* ŽMJ 72 (Figure 1) and *C. jejuni* ATCC 33560 (Figure 2) at the extracts concentrations of 2.5 mg/ml and incubation temperature 37 ºC and 42 ºC, respectively.

B1= A. blazei, PL= P. linteus, LN= L. sulphureus (crude), Lna= L. sulphureus (hot alcali), LP= L. sulphureus (partially purified), KN= G. lucidum (crude), Kna= G. lucidum (hot alcali), KP= G. lucidum (partially purified).

To confirm total inhibition of bacterial growth after prolonged incubation, we carried out growth kinetics test with *P. linteus* crude extract, which showed the best antimicrobial activity in subinhibitory concentrations. *P. linteus* crude extract was added to the growth media in minimal inhibitory concentration determined in broth microdilution test (Table 1). At this concentration it was able to inhibit growth of both, *C. jejuni* and *S. aureus.* However, the extract had better antimicrobial activity against *C. jejuni,* where it displayed bactericidal activity, being able to decrease the number of bacteria below 100 CFU/ml (Figure 4). MIC of *P. linteus* extract showed only bacteriostatic effect on *S. aureus* by keeping its concentration on the starting level (Figure 3).

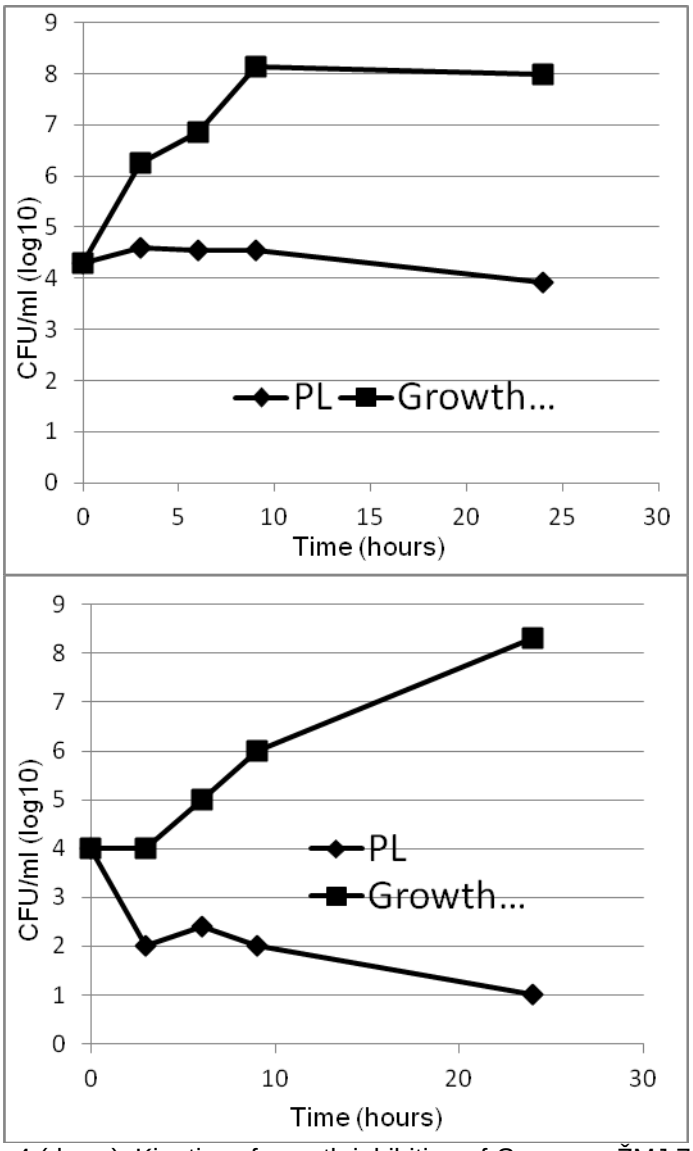

Figure 3 (up), figure 4 (down). Kinetics of growth inhibition of *S. aureus* ŽMJ 72 (Figure 3) and *C. jejuni* ATCC 33560 (Figure 4) at MIC of *P. linteus* crude extract and incubation temperature 37 ºC and 42 ºC, respectively.

In our previous investigations the hot water extract, partially purified polysaccharides and hot alkali extracted polysaccharides showed very high antioxidant properties that have been tested by four antioxidant assays. For *P. linteus* median effective concentrations (EC_{50} values) of the DPPH scavenging activity were low, i.e. < 0.1 mg/ml, while in reducing power and chelating ability of ferrous ions were found to be 0.47 and 0.91 mg/ml. EC_{50} value for inhibition of lipid peroxidation was 7.11 mg/ml (Kozarski et al., 2011). The other tested extracts showed significant antioxidant activity, too, and can therefore be used as a highly effective and valuable food additive, which not only works potentially bacteriostatically, but could also increase the functionality of food product (Kozarski et al., 2011).

This research revealed that crude extracts obtained from *P. linteus* and *G. lucidum,* as well as partially purified extract from *G. lucidum* were effective against *Staphylococcus aureus* and *Campylobacter jejuni*. These data indicate that the tested extracts contain both, antimicrobial and antioxidant components. There was no major difference between antimicrobial activity of tested crude and partially purified extracts, therefore we can conclude that polysaccharides as the main component of the extracts, play the essential role in antimicrobial action against *C. jejuni* and *S. aureus*.

CONCLUSIONS

Crude hot water polysaccharide extracts of *P. linteus* and *G.lucidum* are potential effective bacteriostatical agents against *C. jejuni* in subinhibitory concentrations and at the same time the most effective anti-*Campylobacter* types of mushrooms extracts tested in this study. Further testing in higher subinhibitory concentrations and in food model at lower temperature, as well as sensorial evaluation should be conducted to provide solid evidence for its potential use as a bacteriostatic and functional agent in food.

ACKNOWLEDGEMENTS

The authors would like to thank the Serbian Ministry of Science and Technological Development and the Ministry of Education, Science and Sport of Republic of Slovenia for financing the bilateral project BI-SR-SLO-002-10/11 which enabled this work.

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FOUR STEPS TO RISK ASSESSMENT OF *CAMPYLOBACTER* CONTAMINATION IN BROILER PRODUCTION IN SERBIA

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ABSTRACT: The purpose of the work was to answer some questions important for a qualitative risk assessment in Serbia. The qualitative risk assessment attempts to understand how the incidence of human campylobacteriosis is influenced by various factors during poultry breeding in our country. In the estimation of risks, foour steps are usually involved. 1. Hazard identification: termophilic *Campylobacter spp* are the leading cause of zoonotic enteric illness. 2. Hazard characterization: it is assumed to be dose-independent based on some studies on healthy human volunteers. 3.Exposure assessment: The incidence of *Campylobacter* at poultry farms in Serbia varies from 20 to 80%. Artificial infection of chickens with 6.77 log cfu *C. jejuni* ATCC 29428 per chicken in 21st day of life leads to 5.26 log cfu/g feces after only 5 days. This number has a trend to decrease in next two weeks when 3.02 log cfu/g feces was found. The prevalence of campylobacter-contaminated chickens from positive flock appears to drop from 100% live birds to 50% of chicken carcasses according to our experimental results. The incidence of human campylobacteriosis in Serbia was 4.88 in 2010. 4. Risk characterization: even there is high exposure of human population to *Campylobacter* in Serbia, the incidence of human campilobacteriosis from raw meat is low, mainly because there is a lack of evidence and confirmation of human campylobacteriosis.
Key words: *risk assessment, Campylobacter, poultry*

INTRODUCTION

Risk analysis comprises of three functions: risk assessment, risk management and risk communication. Risk assessment forms the scientific basis for control actions and it is the determination of quantitative or qualitative value of risk related to a concrete situation and a recognized hazard.The food safety risk analysis usually is used as a tool for the control of biological, chemical and physical hazards associated with foods. It is not possible to completely eliminate microbiological hazards but producers are required to protect consumers as far as it is reasonably practicable.

Risk assessment can be quantitative and qulatitative. Despite a number of large and well-publicized quantitative microbiological food safety risk assessment projects, it is probable that the majority of risk assessments in the fields of food safety, health and microbiology are not fully quantitative. There may be a variety of reasons for this. Quantitative microbiological risk assessment is a new and specialized field, methods are still being developed, the expertise and resources to complete them are not widely available, there is a lack of data for quantitative assessment and a lack of mathematical or computational skills and facilities for quantitative risk assessment. A qualitative risk assessment is much quicker and more accessible. Data are required for any type of risk assessment, irrespective of whether qualitative; semi quantitative or quantitative approaches are used. Numerical data are preferred (*Report, 2002*).

Although various foods can serve as a source of food borne illness, meat and meat products are important sources of *Campylobacter*. The *Campylobacter* is most frequently reported cause of zoonotic diseases in EU in 2007, incidence was 45.2 cases per 100 000 population (*EFSA, 2010*). This bacteria is widespread within the poultry production in Europe. *C. jejuni* is frequent commensal in poultry and cattle, and *C. coli* in swine and poultry. The most common way people become infected with zoonotic enteric pathogens is through the

ingestion of food contaminated with animal feces (contamination usually occurs during processing).
An understanding of thermophilic *Campylobacter* and specifically *C. jejuni* in broiler chickens is important from public health.The aim of this paper was to answer some questions important for a qualitative risk assessment in Serbia. The qualitative risk assessment attempts to understand how the incidence of human campylobacteriosis in Serbia is influenced by various factors during poultry breeding and broiler meat production “from farm to table”. In the estimation of risks, the following steps are usually involved: hazard identification, hazard characterization, exposure assessment and risk characterization. The spread of *Campylobacter* in broiler flock was examined in experimental designe: chickens were artificially infected with *Campylobacter* in life period when usually campylobacter infection occurs in poultry; number of *Campylobacter* were examined during next three weeks; chickens were slaughtered on day 42 of life and prevalence of *Campylobacter* on carcasses were examined

MATHERIAL AND METHODS

Experimental design: The control group (group A) consisted of 30 chickens which were not artificially infected with *Campylobacter.* The group B consisted of 32 chickens which were artificially infected with 6.77 log cfu *C. jejuni* ATCC 29428 per chicken on day 21 of life. Total count of *Campylobacter* was examined in chicken feces five times in the following twenty days. The chickens were slaughtered in the poultry abattoir on day 42 of life (10 chickens from the control group and 10 from the infected group). The prevalence of *Campylobacter* on carcasses and livers were determined immediately after processing.
The total number of *Campylobacter* was determined according to procedures of Chen et al. (2003) and ISO 4833. Isolation and identification of *Campylobacter* were performed according to the standard ISO 10272 procedure.

RESULTS AND DISCUSSION

Hazard identification

The genus *Campylobacter* now comprises 17 member species most of which are microaerophils, i.e. grow preferentially in low oxygen concentrations. The majority of cases of campylobacteriosis are caused by two species: *C. jejuni* and the closely-related *C. coli*. These two species are often referred to as the “thermophilic” or “thermotolerant” *Campylobacter* as they grow preferentially at 42°C (EFSA, 2010). Termophilic *Campylobacter spp* are leading causes of zoonotic enteric disease in most developed countries.They are usually indirectly transmitted to humans through the consumption of contaminated food. The principal reservoir of these organisms are the digestive tract of food producing animals. In the vast majority of cases, the organisms are constantly shed in faeces by asymptomatic animals. Handling raw poultry and eating poultry products are important risk factors for sporadic campylobacteriosis.

Hazard characterization

Hazard characterization provides a description of the public health outcomes following infection, including sequelae, pathogen characteristics influencing the ability of organism to elicit infection and illness, host characteristics that influence the acquisition of infection, and food-related factors that may affect the survival of *C. jejuni* in the human gastrointestinal tract (WHO, 2002).
Hazard characterisation includes analysis of data on food pisoning (WHO, 2002):

- consequences
- pathogen characteristics influencing an organism’s ability to cause infection and illness
- host characteristics that influence the acquisition of infection

- food related factors that aneble the survival of pathogens in human gastrointestinal tract.

Food poisoning data usually are obtained after an outbreak but in the case of campylobacteriosis there is no dose-response data from outbreak, because this disease occurs in sporadic cases. Dose-response analysis - the probability of illness following infection is influenced by the immune response of a host and it is assumed to be dose-independent based on some studies on healthy human volunteers (WHO, 2002).

Exposure assessment at farm level

Initial introduction of *Campylobacter* into poultry flock still remains poorly understood and the phenomenon may be multi-factoral. *Campylobacter* usually colonise chickens in the third week of life. The level of colonisation and the spread of *Campylobacter* between animals depend on different factors: breeding conditions, hygiene measures, immune response of animals etc. In food producing animals campylobacteriosis usualy don't have clinical signs. The occurrence of these bacteria varies depending on the range of factors including the organism, geographical factors, farming and meat production practice. Some animal production conditions facilitate the spread of bacteria, such as high density and/or poor infection control *(Stojanov* et al., 2006; *Stojanov* et al., 2009). EU data reported for 2007 indicates that *C. jejuni* was identified in 42.5%, 86.8% and 2.1% of the isolates from broilers, cattle and pigs respectively, while *C. coli* was identified in 13.1%, 9.5% and 87.1% of the isolates from the same animal species (EFSA, 2009).

According to our experimental results artificial infection of chickens with 6.77 log cfu *C. jejuni* per chicken on day 21 of life leads to 5.26 log cfu/g feces after only 5 days, with a tendency to decrease 4.97 log cfu/g (31st day of life), 4.49 log cfu/g (35th day of life) and 3.02 log cfu/g (39th day of life). After this day the increase in *C. jejuni* count was noticed 4.95 log cfu/g (49 th day of life). Tendency of decrease in *Campylobacter* count was found also in the work of Van Boven et all. (2003). The results are shown in Graph 1. In the control group which was not infected, a total of 40 fecal samples were tested and *Campylobacter* was not found.

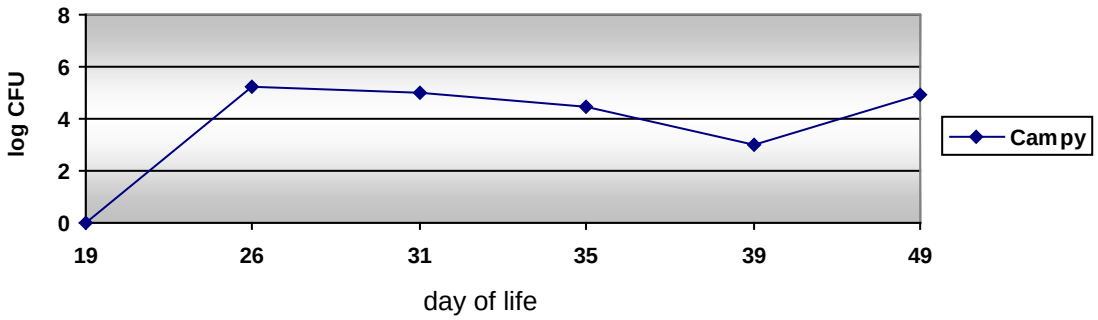

Figure 1. Average number of *C. jejuni* n chicken by days of life (log cfu/g feces)

Exposure assessment at processing

Cooling, freezing, adding of chlorine during water chilling, lactic acid, irradiation may significantly reduce *Campylobacter* contamination of carcasses. According to our experimental results the prevalence of *Campylobacter* -contaminated chickens from positive flock appears to drop from 100% live birds (with 3.02 log cfu/g feces) to 50% of chicken carcasses.These results are shown in Table 1.

Table 1. Prevalence of *Campylobacter* on broiler carcasses

Sample	No samples	Group A control C. jejuni positive (%)	Group B infected C. jejuni positive (%)
Liver	10	0 (0.00%)	5 (50.00%)
Carcase	10	0 (0.00%)	5 (50.00%)
Total	20	0 (0.00%)	10 (50.00%)

These organisms can be transmitted to the production facility and can contaminate the processing environment and the final product. According to our results (Petrović et al., 2007a, b, c) the occurrence of *Campylobacter* is very frequent in poultry carcasses in abbatoirs in Vojvodina region (Table 2). The influence of production management is great, since in poultry abattoirs the prevalence ***varies from*** 11.43 to 90.00% carcasses (Petrović et al., 2008 a, b, c). The prevalence of *Campilobacter* positive carcasses increased during evisceration and decreased uppon the method of chilling.

Table 2: Occurrence of *Campylobacter* in poultry samples (Petrović et al., 2007a)

Occurrence of pathogen (%)		Abattoir mark						
		A	B	C	D	E	F	G
Campylobacter	liver	40.00	5.00	8.56	6.00	34.28	2.86	5.71
	carcasse	90.00	14.28	51.43	20.01	68.57	11.43	31.43

Exposure assessment at disstribution

Cooling is the most important factor that prevents the increase in *Campylobacter* numbers at retail. Frozen chicken posed a lower risk via concumption than fresh meat. In a restaurant and at home cross contamination occcurs rom undercooked and raw meat to other food that is eaten without further cooking. Undercooking is a higher risk than cross contamination. Meat contaminated by *Campylobacter* can either infect humans directly (if not properly cooked or otherwise treated) or indirectly. For indirect contact the contaminated meat acts as a vehicle of *Campylobacter*, especially those in meat juices, which can easily contaminate kitchen equipment such as cutting boards, plates, knives etc. and thereby other foods (salads etc.), which might be eaten without further bacteriocidal treatment (EFSA; 2010).

Exposure assessment-human campylobacteriosis in Serbia

In immunologically-naive humans, the symptoms of *Campylobacter* infection can range from mild, watery diarrhoea to severe bloody diarrhoea with fever, abdominal cramps and an influx of polymorphonuclear leucocytes (dysentery-like disease) (van Vliet and Ketley, 2001). Acute infections may also rarely involve intestinal complications such as appendicitis and colitis, and extra-intestinal infections, such as bacteraemia, hepatitis, abortion/peri natal infection and haemolytic-uremic syndrome (Skirrow and Blaser, 2000). According to the Report of Institute of public health of Serbia »dr Milan Jovanović Batut« In Serbia in 2010 there were 357 cases of *Enteritis Campylobacterialis* diagnosed with the incidence of 4.88, while in the EU in 2005 the incidence rate of 38.2-51.6 cases per 100 000 population was noticed.

Risk characterization

This step links the probability and magnitude of exposure to *Campylobacter* associated with consumption of meat to adverse outcomes that might occur. *Campylobacter* is frequently found in feces of live animals in Serbian farms. Overall *Campylobacter* contamination on carcasses decreased through processing with temporary increases occurring during transport and evisceration. But despite high exposure of population to *Campylobacter* in Serbia, the incidence of human campylobacteriosis from raw meat is low, mainly because there is a lack of evidence and confirmation of human campylobacteriosis. In order to significantly reduce the bacterial load on processed carcasses, interventions would require addressing the bacterial load, both internally and externally. *Enteritis Campylobacterialis* is a sporadic disease; symptoms usually do not require hospitalisation and many sick people do not go to the doctor and only small number of ill people has laboratory confirmation of campylobacteriosis. Also significant factor of exposure to the decrease are cooking habits in Serbia: meat is usually well cooked.

CONCLUSION

In order to significantly reduce the bacterial load on processed carcasses, interventions would require addressing the bacterial load, both internally and externally, since efforts directed at only one of these contamination reservoirs can be readily undermined by high levels of contamination from the other.

ACKNOWLEDGMENT

Project MNTR Government of Serbia no. TR31071 for the period (2011-2014).

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A COMPARATIVE STUDY OF VARIOUS STRUCTURES OF GRINDING PROCESS ON THE HEAD BREAK SYSTEM

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ABSTRACT: Milling is the physical process of converting the endosperm of wheat kernel into flour. The first stage of the modern flour milling process is the break system, in which the wheat kernel is opened up and the contents released so that the endosperm may be separated from the bran. The aim of the study presented in this paper was to investigate effect of various structures of the head break system on the yield and ash content of the streams from the three break systems at the laboratory mill. The conventional milling break system (A), the break system with double high roller mills at B1 and B2 (B), the break system with debranning pre-treatment (C) and the break system with pre-break as a pre-treatment (D), at the similar break release on B1 and B2 were compared. The lower coarse fraction (through 1000-µ and over 630-µ) yield, higher yield of other streams, higher ash content of common streams from three break systems and higher energy requirements for grinding has been obtained using double high roller mill. In the opposite of that grinding with debranning pre-treatment led to increase in coarse fraction yield, decrease in small fractions yield (including break flour yield) and decrease in energy requirements for grinding but ash content of common streams was close to the ones using double rollermill and was higher compared to conventional break system. The most effective break system structure was grinding with pre-break system pre-treatment (3-5% break release through 1000-µ) in which yield of all fractions was close to conventional break system, but with insignificantly lower ash content of all fractions and energy requirements for grinding.
Key words: *grinding, wheat, break system, debranning, eight-roller, break release, pre-break, streams, pre-treatment, yield, ash, energy requirements*

INTRODUCTION

Wheat is one of the most important cereal crops in the world with annual world production of about 650 million tons. According to statistical data from WOH/FAO wheat constitutes about 27% of the total world production of cereals by weight, with maize and rice 35% and 28%. Approximately 70% of wheat is used for food production. Milling is very important in wheat processing. Wheat is milled into flour which is then made into products such as bread, cakes, cookies, cereal, pasta, and noodles. Other uses include the manufacture of alcohol, gluten, and livestock feed.

Flour milling is the process by which wheat is grounded into fine particles and through which the wheat grain is separated into its constituent parts: bran, germ and endosperm. The germ and bran are largely discarded while the endosperm is then further reduced into the fine powder that we call flour.

In principle the milling process is established in three stages, which are break system, purification system and reduction system. The break system is used to break open the wheat kernel and continued to scrape endosperm from the bran, step by step, by sequential passages. The purification system is to separate the outer branny material from inner white endosperm. The aim of this purification system is to purify the milling material that almost no flour is produced. The reduction system is used to deliberately mill the center particles of the wheat grain into flour (Siti Mazlina et al., 2006).

The break system is the beginning of the actual milling process. The break system has two parts: (1) the primary or head break, which releases relatively pure particles of endosperm; and (2) the secondary or tail break, which cleans up the bran and releases smaller pieces of endosperm along with finer pieces of bran and germ (Posner and Hibbs, 1997).

The head break process at most of commercial mill plants consists from three break systems. The objective of grinding on the head break is, at first, to open the whole grain on the first break (B1), and, at second, to obtain as much yield of intermediate-sized particles such as either semolina, middlings and dunst with the lowest of ash content and the lowest energy requirements for grinding.
In recent years, as energy has become increasingly expensive, power costs have assumed much greater importance in the economics of flour mill operations. It is known that as much as 75% of the energy used in a modern flour mill was associated with the actual milling process. The remaining 25% was for processes such as grain cleaning, storage, flour blending, shipping and packaging, and preparation of mill feed (Zwingelberg, 1980).
Emerging trends in recent years are looking for new opportunities and technologies for processing wheat which could decrease energy requirements for wheat milling by improving the grinding process itself by shortening and simplifying. In our recent investigations it was established that at the 20,8 t/h mill the most energy requirements are consumed at the head break stage. About 34% of the total energy is directly used for grinding and 49% for pneumatic lift, respectively (Zhygunov, 2011). This means that the potential for further cost reductions in this stage is high.
As a result, twin grinding stages without any intermediate sifting were applied. It was called a new roller mill design featuring two grinding stages in one machine, known today by the name of eight-roller mill (Baltensperger, 1993, Fišteš, 2009). Another solution is the process of debranning of wheat prior milling. It's a pre-milling treatment that allows a controlled and progressive removal of the grain's layers (Alymkulov, 1979, Satake, 1990). The other solution is the pre-treatment of wheat known as pre-break which is often used in rye milling and widely used in wheat milling at 70-80 (Sukharev, 1983, Dexter, 1986). In recent times, with the trend to shorter mill flows, the prebreak has largely been eliminated.
In the present investigation we report the effect of various structures of the head break system on the milling performance: yields of different fractions of intermediate products, their ash content and energy requirements for grinding.

MATERIAL AND METHODS

Wheat

A hard red winter wheat from the crop of 2009 from south of Ukraine was used. The physicochemical properties of this material were: 12.8% moisture content, 50% glassiness, 1.75% ash content, 12.3% protein content, 38.2 g 1000-kernel weight, 0.74 g/sm^3 bulk density.

Milling procedure

One-kilogram samples of cleaned wheat were tempered to 16,0% moisture content in two stages. At first, the wheat was held in tempering bin for 16 h to reach 15,5% moisture. Then wheat was tempered to 16,0% during 15 min before milling. The mill room was controlled for both temperature (22 □C) and relative humidity (60%).
Conventional milling procedure (model A) was carried out with grinding on B1, B2 and B3 break systems on the laboratory roll stand Nagema which has 220-mm diameter rolls with a fast roll speed of 520 rpm and a slow roll speed of 208 rpm. The effective grinding length is 150 mm. The corrugation of the rolls was 16 cuts per 2,54 cm and they were operated in dull to dull disposition.
For the simulation of the eight-roller milling system on B1 and B2 (model B) the milled stock after the first break was fed to second break without sifting. In a case of debranning procedure (model C) treatment of wheat was carried out using the laboratory pearling mill with an abrasive stone which rotates inside a slotted screen. The wheat sample (500 g) after the first stage of tempering was placed in the hopper of the pearler, with the outlet blocked, and abraded 90 s for the bran ratio of approximately 3%. Polishings removed during the debranning process were determinated by sifting the stock on a 1400 μm screen in a Ro-Tap

sifter for 2 mins. For the pre-break treatment (model D) corrugated rolls with 3-5% break release through 1000-µm screen were used.
The roll gaps were set at 0.54, 0.27 and 0.1 mm for the B1, B2 and B3 breaks, respectively. This way it was possible to obtain 43% break releases (through 1000-µ) both on the first and second break and 33% break release (through 630-µ) on the third break, for the conventional and pre-break models of head break. For the debranning model the roll gaps on the first and second break were adjusted to 0.56 and 0.35 mm in order to obtain the similar break releases on the B1 and B2 according to the ones in conventional model. For the model B only the roll gap on the second break was changed (0.28 mm). Roll gap on the B3 was constant for all models.
After grinding the milled products were sieved for 10 min on a laboratory sifter oscillated at 150 rpm with a throw of 8 cm, using mesh sieves of 1000, 630, 438, 294, and 160 µm along with a bottom pan. Six streams were obtained: overtails (OT, over 1000 µm); coarse fraction (CF, through 1000 µm and over 630 µm); medium fraction (MF, through 630 µm and over 438 µm); fine fraction (FF, through 438 µm and over 294 µm); dunst (DU, through 294 µm and over 160 µm); break flour (FL, through 160 µm). Overtails from the first and second break were milled step-by-step on the next break passages. The same fractions from three breaks were collected (except coarse fraction from third break which with overtails forms a branny product) and the ash content was determined according to the standard method (ICC Standard No.104/1). The total ash content of all milled fractions was calculated from their mass balance.
Watt-hour meter was used to measure the electric energy of the motor. Two different power readings were recorded corresponding to operation with and without the material flow. The no-load energy values were substracted from the values measured during grinding.

RESULTS AND DISCUSSION

The results of intermediate products and flour yield are shown in Table 1. As it can be seen from the results, the highest yield of coarse fraction (1.3 times higher related to conventional milling model) was given using model C. This is because the outer layers serve as external carcass of wheat kernel, and when they were removed debranned wheat kernel becomes more fragile (Kupritz, 1946). In opposite of this fact, the lower yield of coarse fraction and higher yield of other milled fractions has been obtained using double stage grinding on B1 and B2 (model B) compared to conventional break grinding. These results are expected and in consistence with other literature data (Pankratov, 2000). The yield of milled fractions for the grinding with pre-break conditions was similar to conventional grinding.

Table 1. Yield (% w/w) of intermediate products and flour for different structures of head break process

Processing model	CF [a]	MF	FF	DU	BF	Total
Model A	20.40 ± 0.82	15.47 ± 0.05	13.10 ± 0.22	11.83 ± 0.24	18.07 ± 0.37	78.87 ± 0.62
Model B	11.97 ± 0.21	17.90 ± 0.14	16.10 ± 0.62	12.97 ± 0.33	19.07 ± 0.34	78.00 ± 0.92
Model C	26.50 ± 0.78	13.47[a] ± 0.50	11.67 ± 0.45	11.73 ± 0.75	15.17 ± 0.17	78.53 ± 0.37
Model D	20.77 ± 0.29	15.03 ± 0.56	13.63 ± 0.66	11.60 ± 0.14	17.40 ± 0.37	78.43 ± 0.62

Results are given as mean ± standard deviation (n = 3); [a] – collected only from B1 and B2

The results of the ash content, as they are illustrated in Table 2, show the difference in the ash content between the milled fractions from different structures of head break. The ash content decreased from coarse fraction to flour for all samples. The highest ash content of all milled fractions was obtained using model B, the lowest using model D and similar data to conventional model was obtained for the model C. It is possible to assume that the similar dependence may be distributed for the ash content of common flour under industrial conditions (Merko, 1971).

As for the ash content of break flour which was obtained in this investigation it was found to deviate slightly between the flour samples. The ash content of the unpearled flour was only slightly higher (0.02% absolute difference) compared to pearled and such difference could be considered negligible. This is in agreement with other findings that suggest the ash content of pearled flour is approximately similar to that from the unpearled flour, if not slightly higher (Evers et al., 2002; Moss et al., 1998). The findings from this investigation showed that the ash content for the unpearled flour was only slightly higher compared to pearled. The ash content of flour obtained with pre-break on corrugated rolls was the same compared to pearled. The highest ash content was found for break flour obtained with double stage grinding.

Table 2. Ash content (% d/w) of intermediate products and flour for different structures of head break process

Processing model	CF [a]	MF	FF	DU	BF	Total
Model A	1.49 ± 0.04	1.27 ± 0.02	0.85 ± 0.02	0.70 ± 0.03	0.63 ± 0.00	1.03 ± 0.02
Model B	1.62 ± 0.04	1.45 ± 0.04	0.95 ± 0.02	0.83 ± 0.02	0.64 ± 0.00	1.07 ± 0.01
Model C	1.52 ± 0.05	1.30[a] ± 0.05	0.86 ± 0.01	0.65 ± 0.04	0.61 ± 0.02	1.08 ± 0 .02
Model D	1.44 ± 0.04	1.22 ± 0.03	0.82 ± 0.06	0.66 ± 0.03	0.61 ± 0.02	0.99 ± 0.01

Results are given as mean ± standard deviation (n = 3); [a] – collected only from B1 and B2

Table 3 shows the result of the energy requirements for grinding using different head break structures. The highest energy consumption directly for grinding was observed for the model B. It was higher more than 1.07 times compared to the conventional grinding. The energy consumption directly for grinding for both C and D models was almost the same and it was 1.28-1.29 times lower compared to the conventional grinding. However, it was found that the total energy consumption including energy for pre-treatment was only slightly less for the model D whereas the energy consumption for the model C was more than 1.19 times higher compared to the conventional grinding.

Table 3. Energy consumption (KJ/kg) during break grinding for different structures of head break process

		Break system			
Processing model	**Pre-treatment**	**B1**	**B2**	**B3**	**Total**
Model A	—	16.80 ± 0.17	5.76 ± 0.29	5.76 ± 0.29	28.32 ± 0.45
Model B	—	16.44 ± 0.61	7.80 ± 0.61	6.12 ± 0.29	30.36 ± 0.90
Model C	11.64 ± 0.45	12.72 ± 0.17	4.44 ± 0.74	5.04 ± 0.59	33.84 ± 0.59
Model D	6.12 ± 0.29	10.56 ± 0.45	5.52 ± 0.17	5.88 ± 0.34	28.08 ± 0.59

CONCLUSIONS

Five milled fractions were investigated to determine any difference in their weight yield and quality (ash content) due to the structure of head break milling process (conventional, double stage grinding, pre-treatment with debranning and pre-break system). It was found that the different structures of head break process exhibited differences in yield and ash content of milled fractions with the best results for the pre-break grinding. The lowest energy requirements were obtained for this structure of head break, too. The worst results of ash content of milled fractions were found for grinding without sifting after B1 system. The highest energy requirements for grinding were obtained for the double stage grinding as well as for the grinding with debranning prior to milling. However, improvement considering the energy requirements should be expected under industrial conditions because of decreased energy consumption for the pneumatic lift in case of eight-roll grinding, and shortening and simplifying the flow diagram, especially on the tail break stage using debranning process.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project 10/11P “The development of scientific and technical basics for enhanced of flour assortment using energy-saving methods of grain preparation and milling”, financed by the Ministry of Education and Science, Youth and Sport of Ukraine.

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BAKING OF POTATOES AT DIFFERENT OVEN TYPES; CONVECTIONAL AND STEAM ASSISTED HYBRID

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ABSTRACT: Potatoes baking at different types of ovens were studied with respect to some physical and chemical quality criteria of baked sample as a function of baking time. As well as convectional baking, steam assisted baking was utilized in the study. Steam assisted baking is a hybrid method combining the advantages of steam-baking and convectional baking. The shorter baking times provided with steam results in healthier products, with the constitution of harmful compounds at a minimal level. The potatoes by steam assisted baking were produced in a hybrid oven having an inner steam generator. The steam generated from ~350 ml water was injected into the oven cavity totally five times, at five minutes intervals during the baking process. Potatoes baked in steam assisted (steam+forced convection/turbo) and convectional ovens for different baking times (20-70 minutes) was analyzed in the scope of this study. Moisture content, color (CIE a* value, ΔE), texture (hardness, N), sensory evaluation and acrylamide content were investigated. The results of natural and forced convection/turbo baking of potatoes were compared with steam assisted baking, statistically. Acrylamide content of potatoes was found to be related to the oven type; where, the steam assisted oven baking resulted in minimum formation of this compound ($p<0.05$). The sensory points of the potatoes baked at different times was significantly different ($p<0.05$), for all baking ovens.
Key words*: potatoes, steam assisted baking, acrylamide*

INTRODUCTION

Steam assisted cooking of foods is a hybrid cooking method which produces sharing advantages of steam-cooking with natural and/or forced convection baking. It is a healthy cooking method, which provides shorter cooking times with constitution of harmful compounds such as acrylamide, at a minimal level. The increase in society's health standards perspective has resulted in an increase of interest on steam and/or steam assisted cooking of foods.

Desirable quality of thermally-treated potatoes includes proper color, taste and texture. Abnormal colour and excessive softening or firming cause rejection of the product by a consumer (Nourian et al., 2003). During the process of thermal treatment, the composition of the potato changes. The kind and extent of those changes connected with the starch affect the quality of thermal treatment and the quality of potatoes especially textural quality after that process. Texture of cooked potato was investigated by many researchers (Thybo et all., 1998; Truong et all., 1998; Binner et all., 2000; Kaur et all., 2002; Van Dijk et all., 2002; Abu-Ghannam ve Crowley, 2006; Chiavaro et all., 2006; Garcia-Segovia et all., 2008).

Recent studies have reported significant levels of acrylamide in heated foods, most especially, carbohydrate-rich foods (Rosen and Hellenas, 2002; Tareke et all., 2002). The researchers reported moderate levels of acrylamide (5–50 ppb) in protein-rich heated foods and higher content (150–4000 ppb) in carbohydrate-rich foods such as potato. Simple cooking experiments showed that different cooking practices can dramatically affect the acrylamide content of foods. Acrylamide content can be correlated to food browning, although this may or may not be a causal relationship. The method of cooking significantly affects the acrylamide content of foods. For instance, grilling of potatoes produced higher levels of acrylamide than either frying or oven baking (Ahn et al., 2002).

In this study, in order to obtain healthy cooking with low acrylamide content and at the same time to protect the quality features of potatoes, steam assisted (steam+forced convection) hybrid oven was used to cook potatoes as a comparison to conventional cooking. Potatoes were cooked at 210°C for different baking times (10, 20, 30, 40, 50 and 60 minutes) and moisture content, colour (CIE a* value, ΔE), texture (hardness, N), sensory evaluation and acrylamide content of potatoes were investigated for natural convection, forced convection and steam assisted hybrid ovens.

MATERIAL AND METHODS

Potatoes (*Granola*) were stored at 10ºC for maximum 5 days in a dark room after harvesting. The skin of the potatoes was removed before the oven cooking. Cooking experiments were carried out in a steam assisted oven (Blomberg) and also in a domestic use electrical convectional oven (Blomberg, BKO 9566). Steam assisted oven used in the study is a hybrid oven with 48×43×25 cm dimensions having an inner steam generator mounted at the back panel of the oven. The steam generated from ~350 g water was injected into the oven cavity in five times, at five minutes intervals during the cooking process. For the potatoes cooked at different times at 210°C, the physical quality; (moisture content (w/w, wb), color (a* and ΔE value), hardness (N)) as well as acrylamide content (ppb) and sensory quality were determined. The comparison has been made with the results of natural and forced convection/turbo cooking of potatoes, statistically.

Moisture content and colour measurement

The moisture content of potato samples was determined by gravimetric method under vacuum at 70ºC for 24 h as two parallel (AOAC, 1990). The surface colour of the potato samples (a*, b*, L*) was measured by CIE-Lab and ΔE values of cooked samples were calculated from the following equation (CIE, 1978);

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \qquad (1)$$

Texture analysis

Texture analyses were achieved in texture analyser (TA-XTPlus, Stable Microsystems, Surrey, England) using 2 mm needle probe with 5 kg load cell. The test parameters are pre-test speed of 1.0 mm/s, test speed of 1.5 mm/s, post-test speed of 5.0 mm/s, distance of 6 mm and trigger force of 5 g. The hardness was determined from the maximum peak force (N).

Acrylamide content determination

Determination method of acrylamide content of potatoes includes extraction with water, addition of D_3 acrylamide as an internal standard, bromination and GC-MS analysis in the selected ion monitoring (SIM) mode. Bromination of acrylamide to 2,3-dibromopropionamide (2,3-DBPA) was achieved using potassium bromide and potassium bromate under an acidic condition. The operating parameters for GC-MS analysis were as follows: oven temperature was 65℃ (for 1 min), then increased to 240℃ by ch anging temperature by 15℃/min, the temperature was maintained at 240℃ for 10 min, inj ection block temperature was 250℃ and transfer line temperature 240℃. In the SIM mode, m /z 149 for 2,3-DBPA and m/z 153 for internal standard were used for quantification (Tareke et all., 2002).

Sensory evaluation

All sensory tests were performed by a semi-trained panel using uni-polar and bi-polar scale with 8 anchor to determine following attributes: crust redness (1=absent, 8=extreme), brightness (1=pale, 8=bright), hardness (1=extreme soft, 8=extreme hard). Also overall

acceptance were determined using 1-5 scale (1=unacceptable, 2=poor, 3=acceptable, 4=good, 5=excellent).

RESULTS AND DISCUSSION

It was resulted that moisture content of potatoes cooked in steam assisted oven was higher than that of cooked in natural and forced convection, statistically ($p<0.05$). The moisture content of the potatoes decreased with increasing cooking time (Fig 1).

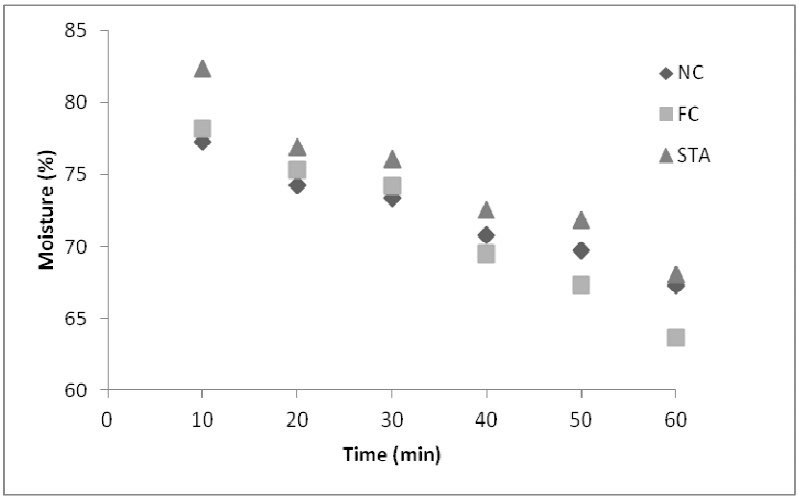
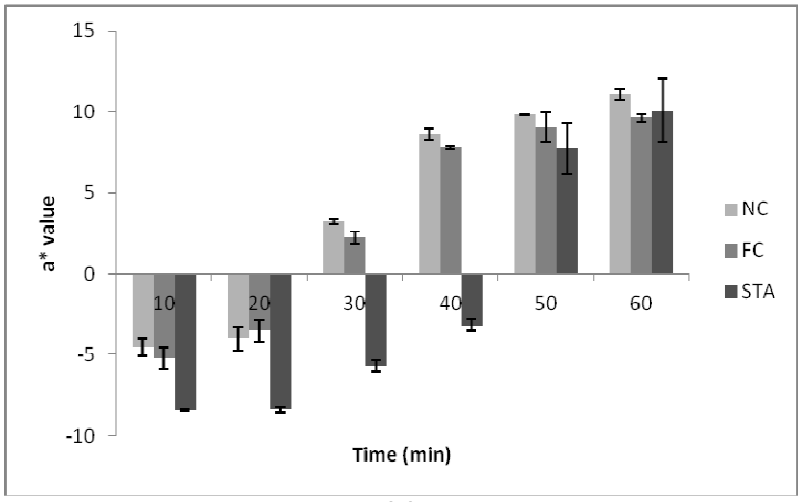
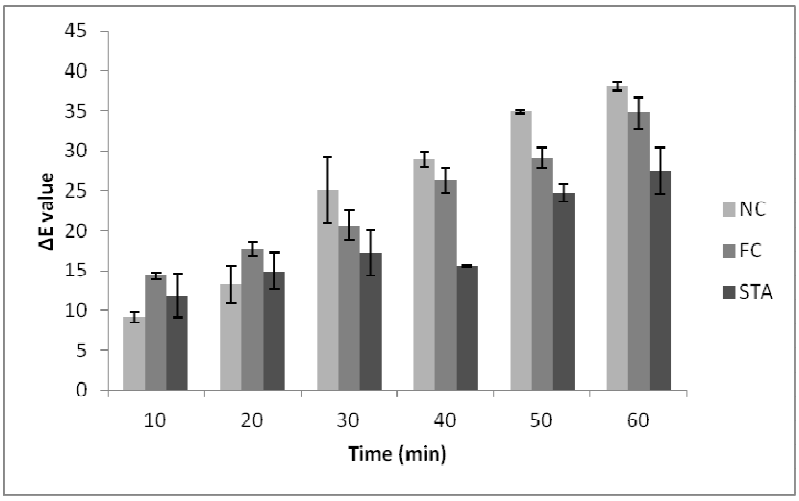


Fig 1. Moisture content of potatoes (wet basis) cooked at natural convection (NC), forced convection (FC) and steam assisted hybrid oven (STA)

Colour change of samples (ΔE) baked in natural and forced convection ovens was higher than that of baked in the steam assisted oven. This result indicated that radiation mechanism was more responsible for colour change in convection baking. This result was supported by a* values of potatoes cooked in natural, forced convections and steam assisted ovens which are illustrated in Figure 2. ΔE values were lower for steam assisted cooked potatoes ($p<0.05$).



(a)



(b)

Fig 2. Color values of potatoes cooked at natural convection (NC), forced convection (FC) and steam assisted hybrid oven (STA), (a): a* values, (b): ΔE values

The steam assisted cooking resulted softer potatoes than those of cooked in the convectional ovens (Fig 3). Softening increased with increasing cooking time for all oven types. However, in steam assisted hybrid oven heating rate increased due to steam in the oven chamber and this phenomenon caused an increase of softening of potatoes cooked in this type of oven.

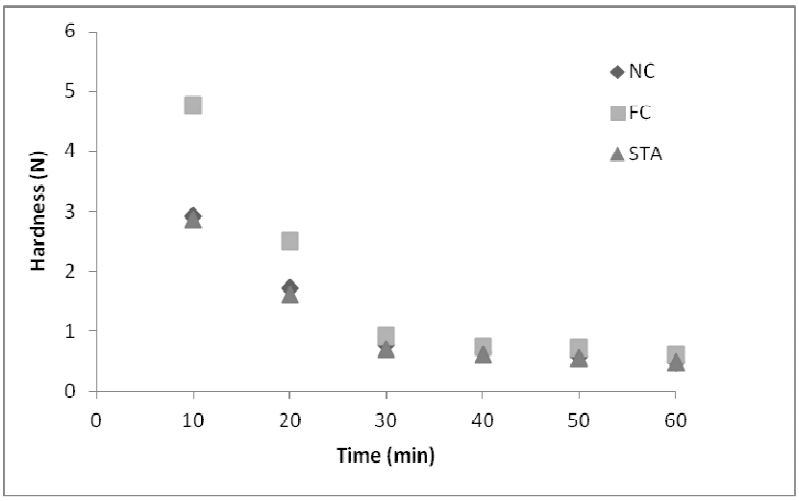


Fig 3. Hardness (N) values of potatoes cooked at natural convection (NC), forced convection (FC) and steam assisted hybrid oven (STA)

Acrylamide content of the potatoes was found to be related to the oven type ($p<0.05$), the forced convection oven baking resulting in the maximum formation. Steam assisted baking caused a reduction in the acrylamide formation compared to the natural convection and forced convection oven baking (Fig 4).

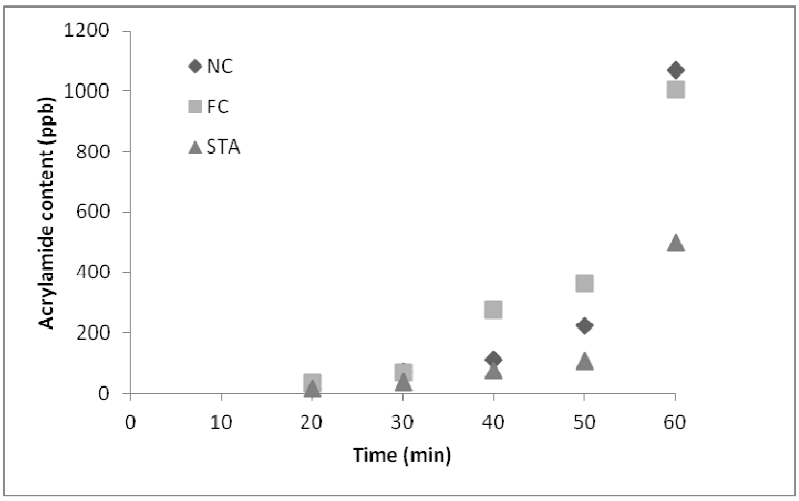


Fig 4. Acrylamide content of potatoes cooked at natural convection (NC), forced convection (FC) and steam assisted hybrid oven (STA)

Sensory evaluation results of cooked potatoes are illustrated in Table 1. Crust redness of samples was determined higher in convectional ovens whereas crust brightness was higher in steam assisted hybrid oven. It was observed that hardness values of potatoes cooked in steam assisted hybrid oven were lower, those were in line with instrumental hardness values. Overall acceptance of steam assisted cooked potatoes was also high.

CONCLUSIONS

Steam assisted oven cooking can be concluded as a good choice over natural convection or forced convection cooking in that it was resulted in lower acrylamide content, lower ΔE value and high overall acceptance as result of sensory evaluation. In addition steam assisted oven may offer shorter cooking time because of yielding fast softening of potatoes.

ACKNOWLEDGEMENTS

This research was supported by the Scientific and Technical Research Council of Turkey (Project No. TOVAG 109 O 087). Also, equipment and material support of the companies of ARCELIK and Menemen Ege Agricultural Research Institute was acknowledged.

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Table 1. Sensory evaluation results of cooked potatoes at different oven types

Time (min) /sensory parameter	crust redness			crust brightness			hardness			overall acceptance		
	NC	FC	STA	NC	FC	STA	NC	FC	STA	NC	FC	STA
10	2.0 (±0.1)	1.6 (±0.1)	1.6 (±0.1)	5.4 (±0.1)	5.2 (±0.1)	6.9 (±0.1)	5.9 (±0.1)	6.2 (±0.1)	5.1 (±0.1)	1.5 (±0.1)	1.56 (±0.1)	1.9 (±0.1)
20	2.9 (±0.1)	3.1 (±0.1)	2.6 (±0.1)	4.5 (±0.1)	4.0 (±0.1)	6.1 (±0.1)	5.1 (±0.1)	4.9 (±0.1)	4.7 (±0.1)	2.9 (±0.1)	2.6 (±0.1)	3.2 (±0.1)
30	3.6 (±0.2)	3.6 (±0.0)	3.3 (±0.1)	2.9 (±0.1)	3.1 (±0.1)	4.9 (±0.1)	4.0 (±0.1)	4.1 (±0.1)	3.4 (±0.1)	2.9 (±0.2)	3.3 (±0.1)	3.6 (±0.3)
40	5.8 (±0.1)	5.3 (±0.0)	4.7 (±0.1)	2.8 (±0.1)	2.7 (±0.1)	3.7 (±0.1)	3.7 (±0.2)	3.8 (±0.1)	2.8 (±0.1)	4.2 (±0.1)	3.7 (±0.1)	3.7 (±0.1)
50	6.1 (±0.1)	6.2 (±0.1)	5.5 (±0.1)	2.5 (±0.1)	2.9 (±0.0)	3.2 (±0.1)	3.1 (±0.1)	3.1 (±0.1)	2.5 (±0.0)	3.0 (±0.1)	3.1 (±0.1)	3.2 (±0.1)
60	6.7 (±0.1)	6.4 (±0.1)	6.1 (±0.1)	1.9 (±0.1)	2.1 (±0.1)	2.6 (±0.1)	2.7 (±0.1)	2.9 (±0.1)	2.3 (±0.1)	2.4 (±0.1)	2.2 (±0.1)	2.6 (±0.2)

NC: natural convection, FC: forced convection, STA: steam assisted hybrid oven

BENEFIT OF MOISTURE REMOVAL FROM FEED MANUFACTURED SOLIDS WITH VACUUM TECHNIQUE AND ITS COMPARISON TO TRADITIONAL AIR COOLING METHOD

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ABSTRACT: Vacuum dehydration technique (VDT) is known as a rapid evaporative technique for moist and porous solid products. Known advantages of VDT is short processing time, extension of products shelf life and improvement of product properties related to quality and safety issues. In this study, animal feed wheat-based diets with different particle size distributions, ground by hammermill, through 1, 3 and 5mm screen size was manufactured through different pellet-die hole diameters (2, 3.5 and 5mm). Moisture was removed by traditional air dehydration technique (ADT) or VDT. Measuring the dry matter was done immediately after the dehydration of feed pellets. Pellet durability index (PDI %) and hardness analysis were done 30 days after the dehydration. Successive dynamics of water loss was performed better while using the VDT compared to ADT where VDT appeared to reach the industrial moisture equilibrium much faster. It was not observed a clear pattern of influence on dehydration efficiency of different feed structure represented by particle size distribution. The volume increase of the product confirmed to have negative influence on moisture removal from feed pellets when conventional ADT was subjected whereas no clear outline was confirmed for VDT. This explains that increase of feed pellets diameter can limit the moisture removal for both, VDT and ADT. The VDT has demonstrated positive influence on PDI when compared to traditional ADT. Pellet hardness analysis didn't show clear influence of the different dehydration techniques on manufactured feed solids.
Key words: *vacuum, air drying, feed moisture, dehydration, animal feed, durability*

INTRODUCTION

At a given temperature and pressures, molecules of the moist solids vibrate in a random and chaotic way and the water molecules are getting easily released from solids in the gaseous state. At a rate that depends on temperature and the state of binding at the surface, water molecules may break their bonds, leave the solid surface and enter the gaseous phase (Chambers et al., 1998). In porous feed solids, an internal mass transfer may occur through the solid part or within the void spaces (Karel, 1975). Dehydration technique (DT) used on feed solids differs by the heat transfer properties. Moisture-containing solids can be dehydrated by subjecting them to reduced pressures, better known as the vacuum. Vacuum treatment can be defined as an act to reduce the density of a gas in a vessel to a value adequate for its planned purpose. First known commercial vacuum product dehydration was performed in 1948 for reducing field heat of lettuce (Thompson and Rumsey, 1984). All recent research advances have confirmed that vacuum technique is a method to shorten processing time and improve product quality (Zheng and Sun, 2004). Many factors affect the speed and efficiency of the product being dehydrated by vacuum technique. These factors are: the surface area exposed to the atmosphere (Do et al., 1999), specific heat of the product prior the dehydration (Haas and Gur, 1987), density (Cheyney et al., 1979; Krokida et al., 2000) as well as porosity (McDonald and Sun, 2001a,b; Tsami and Katsioti, 2000) and particle size of the product (Krokida et al., 2000).

Different moisture removal rate between conventional ADT and VDT is caused by differences in dehydration mechanisms (Sun and Wang, 2000). Air dehydration is principally achieved through heat transfer (conduction and convection), while vacuum dehydration is managed by mass transfer, or water evaporation in the micro pores and at the surface of the product. Vacuum dehydration is caused by pressure difference between the saturation pressure on the walls of the micro pore and diffusion of water vapor through the pore spaces to the surface of the moist-solids and surrounding atmosphere (Wang and Sun, 2002b). In VDT the porous structure plays important role in determination of water evaporation (Zheng and Sun, 2004). Important factor in VDT is that a manufactured solids are being dehydrated in controlled atmosphere which gives advantages as prolonging the shelf life (McDonald and Sun, 2000). The steam is the important ingredient for manufacturing the animal feed, among the other reasons because of the given hydrogen bonds which are connecting various particles of mixed ingredients with the help of adhesion process (Kinlock, 1987). Moisture content and its type play an important role in these processes (Behnke, 2001). Close contact between the wetting adhesive molecules (water) and the solid surface of the feed mash particles give rise to attractive forces known as physical intermolecular interactions (Van der Waals forces). In this way different size of macromolecules in the feed solids inter-diffuses and creates an interpenetration layer in the capillary microstructure capable to bear mechanical load (Possart, 2005). In such microstructure there are some driving forces for the mass transfer of water molecules defined as pressure gradients and capillary forces. The exchange of a gas or liquid in vacuum environment is driven by a pressure difference (Fito and Chiralt, 2000).

Moisture removal by VDT presumably can decrease the time for such requiring operation in the feed production. Moisture removal from feed pellets in vacuum environment can influence positively on intermolecular bonding, thus assumable better physical properties of the feed pellets might occur. The exhaust air from cooler and dryer used in the feed industry is usually highly contaminated with fine powder carry-over which must be run through a cyclone separator to recover the carry-over and also an exhaust air filter in order to reduce the air pollution to an acceptable level. This can potentially create an unstable product quality. With the VDT these problems can be avoided. Advantages of the animal feed dehydration by VDT might meet the quality control requirements much easier than ADT. So far, no detailed experimental study has yet been done for determining the influence of VDT on the quality of feed solids and its final moisture. The current experiment was undertaken to test the assumption that different texture, volume and surface area of the animal feed products have an influence on the moisture removal and the physical quality by VDT and ADT.

MATERIAL AND METHODS

Nine feed experimental diets were produced by pelleting technique at the Center for Feed Technology (Fôrtek) at the Norwegian University of Life Sciences (UMB), Ås, Norway. Major feed ingredient in the experimental feed formulation was wheat and the formulation was typical broiler feed diet. Wheat was milled in Bliss hammer mill (Bliss Industries, Inc. Oklahoma U.S.A. Model E-22115-TF) fitted with 1, 3 and 5-mm screens with the constant energy consumption of 15 amps. The hammermill chamber was equipped with two screens and was driven by 18.5kW electric motor with a rotational speed of 3000 rpm. Air was sucked through the hammermill screens by the speed of 7.2 m/s with the use of a Jesma Co (Sprout Matador A/S, Esbjerg, Denmark) fitted with a type DFC filter. Mixing of all experimental diets was done in a twin-shaft paddle mixer Dinnissen (Pegasus Menger 400 l, Sevenum, Holland) for 180 seconds. Prior to production, apparent bulk density of a mash was measured by filling up the mash in 1000 ml beaker by the free fall, in order to ensure the same production output (table 1). The production output for all the diets was 1100 kg/h. The temperature of the feed mash prior production for all diets was 20 ℃. The feed-mash was conditioned for 30 seconds prior pelleting. Conditioning temperature was 75℃ (± 1 ℃) in a continuous conditioner with the steam produced at 5.4 bars and 153.23℃ by fire-tube boiler (Peder

Halvorsen A/S, Norway, No. 18388, 1997, Model: PH Kvikk-600 kg/hr, max.10 bar) and steam pressure regulated down to 2.5 bar by steam regulating valve (Gestra 1,1 – 10 bar, AG type 5801, F-340, Bremen, Germany) prior pelleting process. The ring-die pellet press (Munch Wuppertal, Germany, 1.2 t/h, 2×17.5kW, RMP350.100) was used. The die hole diameter of the pellet dies used for the experiment was 2, 3.5 and 5 mm. Rolls distance from the die inner-surface was set on 1mm for all the dies used for this experiment. Pellet length and diameter was set proportionally to pellet diameter (table 1).

Table 1, Feed pellet length, diameter, sieve size used before and after holmen durability analysis and the feed mash densities

Pellet diameter (mm)	Pellet length (mm)	Sieve size used before & after Holmen durability analysis[1] (mm)	Feed mash milling particle size and densities (HM- hammermill) (kg / liter)
2	8	1,6	**HM 1mm** - 0,59; **HM 3mm** - 0,61; **HM 5mm** - 0,68
3,5	14	2,8	**HM 1mm** - 0,59; **HM 3mm** - 0,61; **HM 5mm** - 0,68
5	20	4	**HM 1mm** - 0,59; **HM 3mm** - 0,61; **HM 5mm** - 0,68

[1] *Pre-sieving of all the experimental pellet samples was done to give us precise weight of pellets without any dust, thus more precise results for holmen durability analysis. Sieving was run for 30 seconds with vortexing amplitude of 1.5 mm prior and after the holmen durability tests. The size of the sieves for pre and post holmen durability analysis sieving was chosen upon the pellet diameter.*

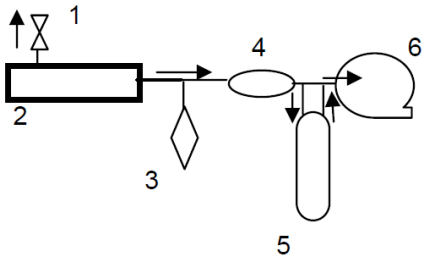

Figure 1, The schematic diagram of the research vacuum moisture removal apparatus:

(1) Pressure release valve (2) Vacuum chamber (3) Pressure control valve and barometer, (4) Steam condenser, (5) Air filter, (6) Vacuum pump

Two different DTs, VDT and ADT, were used immediately after the production of feed pellets. Conventional air flow (ADT) was used for all the diets with the same volume of feed pellets in the batch coolers (UMB - Center for Feed Technology, Ås, Norway). The average air speed in the batch coolers was 1,5 m/s created by the fan (W2E 300-DA 01, W-160) with the air volume capacity of 2550 m³/h. For moisture removal by VDT the research vacuum moisture removal apparatus (figure 1) was used. In the vacuum chamber a residual pressure of 200 mbar was used. A preset heated vacuum chambers wall had a temperature of 75 ºC for all the diets in order to avoid condensation of water on the vacuum-chambers inner-walls. Temperature was measured by digital thermometer (Arnitsu-M, HA-250k; Arnitsu meter co.ltd., Japan).The dwell time for dehydration of the feed was five minutes for all the diets and both DTs. After the moisture removal by VDT the vacuum pump was switched off and the air was re-admitted into the chamber very fast in order to avoid the back-return of any potential remaining condensate from chamber into the pellets. For ADT the air flow was stopped by switching off the fan. All experimental representative feed pellet samples were placed into the sealed plastic bags and stored immediately on the constant temperature of -18℃ for 30 days prior the analysis. Dry-matter analysis was performed in the oven (Termaks) with the standardized procedure (EU 71/393). Thereafter, duplicate samples were weighed, and the differences between the wet and the dry weights were given as calculated mean for moisture level. Durability of pellets was measured using the holmen durability tester (Holmen Chemical Ltd., Borregaard Group, Norsolk, UK), where 100 g of pre-sieved feed pellets, without any dust, were conveyed pneumatically in a closed circuit for 60 s, followed by sieving through a sieve size chosen proportionally to feed pellet diameter (table 1). PDI

(%) was recorded as the proportion of feed not passing through the given sieve size after the stress applied by holmen tester. Durability test was run as duplicate. Hardness analyses defined as the maximum force needed to crush a pellet were performed by Kahl cylinder tester. Methods have been described in detail by Thomas and Van der Poel (1996). The uniformity of feed pellets ready for the analysis (30 per sample) was recorded with an electronic digital caliper (Würth 0-150mm) before the hardness analysis. The experimental data were partially subjected to t-test analysis to examine possible effects of the dehydration methods on the responses, feed moisture and the physical pellet properties. Software used for descriptive and inferential statistics was SAS (SAS Institute Inc., 1999). Significant differences between treatments were determined by using the GLM procedure, LSD t-test. The Unscrambler (CAMO Software AS, Norway) was used as a tool for complete multi-factorial data analysis to examine possible effects and interactions between the production factors on the responses as well as an interactions between the responses.

RESULTS AND DISCUSION

Comparison of the results obtained from usage of different DTs and its influence on physical properties of feed pellets with the results from the literature was difficult due to differences in product composition, production technology, treatment and the structure of the product.

Influence of the feed structure, DT and volume of the feed pellets on dehydration rate and physical quality of feed pellets

No notable changes were seen in process parameters during the feed production to rationalize the differences in moisture content and PDI for all the diets. That should be addressed to experimental production parameters as pellet diameter, length, particle size distribution and DTs.

The dehydration rate during the ADT is initially dependent on the evaporation rate of unbound water at the surface of the feed solids where water needs to diffuse between the feed particles to its dry surface in order to be removed. Yet, during the VDT critical moisture content was easily reached in the vacuum conditions where the moisture loss was balanced within the entire volume of the product. This has shown that dehydration in vacuum conditions is faster while at the atmospheric pressure and air flow slower. Results presented in table 2 and figure 2 shows that the DT has major influence on moisture removal.

Milling with the hammer-mill and the screen size-set of 1, 3 and 5 mm showed to have different particle size distribution with the major particle size mean between 0.2 and 0.5 mm. The mobility of water in feed pellets is shown to be dependent of the pore structure and that is in agreement with Van Brakel and Heertjes (1974) (table 2). The mechanism of DT during VDT showed to be influenced by the difference in particle size distribution and larger pellet volume. However, a clear pattern for ADT was not observed. For more dense feed pellets, with small void space between the particles (HM 1mm) the quantity of water, existed as the vapor which takes part in diffusion, showed better results than in less dense feed pellets (HM 3 and 5mm). That is not in correspondence with findings from Waananen and Okos (1996). In 5 mm pellet diameter no clear difference was observed between particle size distribution obtained by grinding through 5 mm and 1 mm hammer mill screen. Possible reason for this might be an unstable steam quality while feed mash was conditioned hence different adsorption between the particles.

Table 2, Influence of dehydration techniques on moisture content and physical properties in the feed product 5 minutes after dehydration (means and *p* values)

Test name [1]	2mm HM 1	2mm HM 3	2mm HM 5	3.5mm HM 1	3.5mm HM 3	3.5mm HM 5	5mm HM 1	5mm HM 3	5mm HM 5
Product moisture (%),VDT	11,5	11,61	11,8	12,43	13,03	13,34	13,6	12,67	13,58
Product moisture (%), ADT	12,44	12,49	13,25	13,49	13,54	13,7	13,25	12,92	13,51
Moisture (%) – *p*-value, ADT VS VDT	0,002	0,035	0,011	0,003	0,018	0,049	0,75	0,108	0,431
[2] PDI %, VDT	91,79	89,3	91,11	93,22	91,67	84,69	90,42	85,5	78,83
[2] PDI %, ADT	89,6	86,13	88,12	92,08	89,6	82,7	89,13	83,31	72,77
[2] PDI % - *p*-Value, ADT VS VDT	0,041	0,015	0,0001	0,022	0,0001	0,026	0,046	0,043	0,01
Hardness (kg), VDT	3,32	3,33	3,3	5,11	4,84	4,16	7,71	6,17	6,05
Hardness (kg), ADT	2,67	3,09	3,09	4,95	5,03	4,2	7,07	5,8	4,93
Hardness (kg) *p*-Value, ADT VS VDT	0,002	0,108	0,347	0,748	0,687	0,944	0,074	0,299	0,477

PDI and moisture means are derived from the diet and its replica statistically analyzed (P<0.01) in a GLM procedure - LSD t-test
[1] 2.5; 3.5 & 5 mm indicates the pellet diameter
[1] HM 1, 3 & 5 indicates hammer mill grinding with the screen hole size of 1, 3 & 5 mm
[2] PDI (%) indicates Pellet Durability Index % (a predictor of pellet fines produced during mechanical handling)

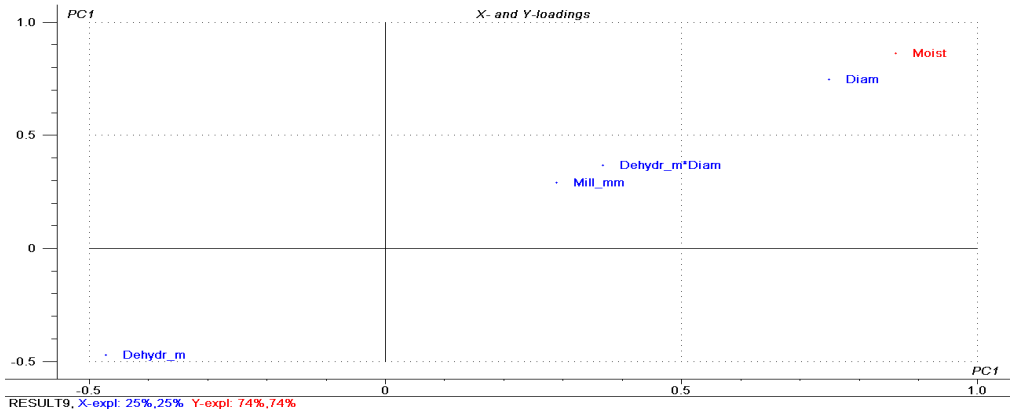

Figure 2, Unscrambles Partial Least Square (PLS) I regression. The DT (Dehydr_m) shows the greatest influence on diagonally positioned moisture (Moist) when compared to other factors.

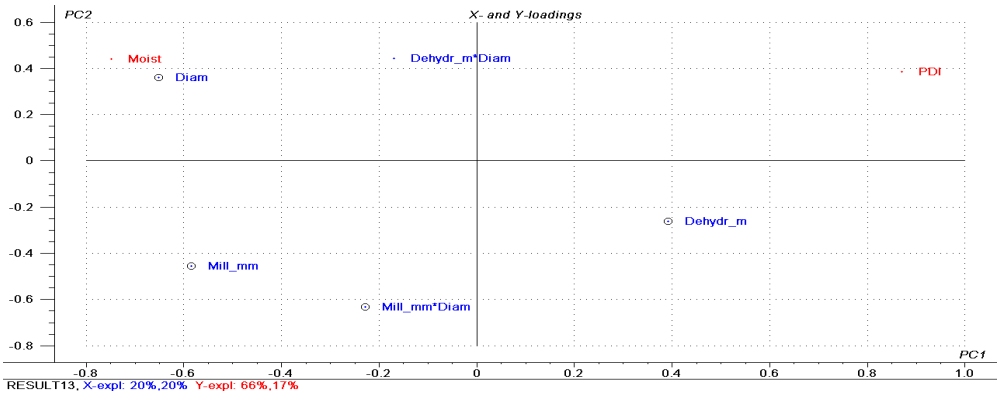

Figure 3, Unscrambles Partial Least Square (PLS) II regression test with jack knifing. The black rings not positioned in the same squared field in the figure assign what factors are influencing moisture and PDI. No influence of products moisture content on PDI %.

As Xiong et al. (1991) have postulated, the binding energy at high moisture levels is small and increases as the moisture content decreases. Since moisture was removed faster with VDT this can partially explain the enhanced physical properties of feed pellets dehydrated by vacuum. In order to acknowledge that moisture did not have any influence on PDI, partial least square regression II test with jack knifing was performed (figure 3). The Unscramblers model for X-variable (dehydration technique, milling, pellet diameter, interaction between particle size distribution and pellets diameter as well as dehydration method and diameter) was used against both outputs, PDI (%) and moisture (figure 3). Durability of feed pellets showed to be positively influenced by DT or precisely, when VDT was applied (table 2, figure 3). This can be explained by allowance of rapid transfer of moisture in the gaseous state to vacuum environment where structural collapse is prevented as suggested by Lin et al. (1998). During the ADT the increase of porosity was supposedly influencing on weakening the inter-particle links inside the feed pellets. Considerably better durability of feed pellets after using the VDT presumably can be explained by rapid decreasing of the moisture content where vapor is no longer in equilibrium with the liquid and the rate of vapor desorption becomes rate-controlling which is a consequence of high binding energy at low moistures. Second assumption is that polymerized network among the feed nutrients and its complexes were set by VDT from chaotic into the organized network. Therefore the greater durability of feed pellets was observed. This phenomenon was explained by Bistac and Galliano (2005), when the arranged network increases, macro-friction decreases due to chain orientation phenomena. Therefore, the effects of elastic contact and bulk dissipation are minimized and the interfacial adhesive behavior of feed components is magnified. Molecular orientation during the adhesive interactions at the surface must be mentioned, where the cracks on pellets might be recovered by VDT and hence better physical properties achieved through formation of attractive properties within the cracks in the polymerized feed structure. Also, better physical properties occurrence by usage of VDT might be explained on the molecular level where the adhesion on polymer fiber interface can be improved by static generation of the low-stress matrix, which will all potentially lead to high-bond strength. Considering that the durability of pelleted feed was increased by VDT, it can be concluded that vacuum has assisted mechanical and chemical adhesion of feed components after the pelleting process with dispersive (van der Waals) forces and diffusive adhesion where the surface pressure of the liquid vapor was decreased and surface energy increased. On the other side, the physical properties defined by hardness analysis didn't seem to be influenced by DTs when statistically analyzed by LSD t-test derived from the GLM procedure.

CONCLUSIONS AND SUGGESTION

Shown results indicate that a VDT can be used for the moisture removal from the animal feed pellets. The advantages of VDT are several. Most important is that a feed product can be dehydrated in shorter time. VDT has already demonstrated to provide many benefits to the food processing industry as shortening product hold up time, increasing production throughput, reducing energy consumption and minimizing microbial growth. Furthermore, unlike conventional air flow dehydration, by using the VDT it is easier to remove large proportions of water molecules in the short time span, which makes itself a much more advantageous method for products of high water content, especially in the extruded fish feed and pet food. After the extrusion the fish feed is subjected to the high air temperatures for a short time where the thermo-labile components with the excess water content, as astaxanthin, and vitamin E would be damaged. VDT might be used here as a hybrid DT composed of complementary drying which donate advantages of dehydration of extruded fish pellets on lower temperatures where anti-oxidative components can be preserved. In addition to the above, the VDT is a more hygienic process since the air goes only into the chamber at the end of the cooling process when the chamber is open to release vacuum, thus the hazardous material level can be diminished. As VDT is carried out in a static state, there is no mechanical load thus damage as in some other DTs. Regarding the apparatus for VDT, the vacuum chamber should include some mixing equipment for rearranging the position of product in order to prevent uneven product dehydration. The chamber must not allow condensation of water on its interior and that's why should be heated. In case of feed pellets, such condensation can cause the product to be unevenly dehydrated. High share mixing can damage the product being dehydrated, therefore slow speed twin-shaft mixing elements should be set in the vacuum chamber with the evaporator. However, modeling of VDT for the feed pellets is still largely unexplored and requires further research. Optimal method should be selected according to a few factors, such as: the economy, convenience, utilization of equipment, operating conditions, personal preference and product requirement. The cost of VDT is comparable with the cost of other DTs even though the initial capital investment might be high. The VDT might make a significant contribution to better quality and easier feed manufacturing.

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BRIQUETTING AS A WAY OF UTILIZATION FOR WASTE BIOMASS

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ABSTRACT: Technology briquetting is the process by which the crushed material under high pressure is converting into a compact form of high density.
Because of the variability of biomass consisting of: wheat, barley, oat, rye straw, maize, oilseed straw, sunflower stalks and remnants of shells, fruit and pruning of vineyards, forest biomass, waste wood in wood processing machine consists of a complex technological process of production of briquettes.
Contemporary machine-technological solution for the biomass briquette plant is a complete solution that comprises following processes: collection, transportation, drying, grinding, briquetting, and storing and product delivery. Process of transferring biomass into briquettes has a significant positive contribution to environment protection.
Key words: *briquetting, waste biomass, environment protection*

INTRODUCTION

Briquetting technology is the process by which the chopped or grinded material under a high pressure is converting into a compact form attributed with high bulk density. According to Zubac et al. (1988) to be able to convert biomass to briquettes raw material should meet following criteria: moisture content should be in the range 10-18%, particle size not greater than 10 mm and briquetting temperature of 70-90^{o}C (Zubac, 1988). The most common practice is that biomass should undergo extra drying and volume is reduced by 7-12 times. Due to the diversities of raw material mainly consisting of wheat, barley, oat or rye straw, cornstalks, hay crops, residue of sunflower stalks and husks, pruning fruit and vineyards, forest biomass, wood waste in wood processing etc., a complex machine technological procedure is involved in briquette making (Brkić and Janić, 2008).

MATERIAL AND METHODS

The importance of briquette making, in particular, has increased in recent days, when there has been experienced the increased shortage and price increase of oil and gas as conventional fuels. At the beginning, bio briquettes were mainly experienced for solving problems of waist surplus in some production areas like wood processing and others. Those industries used briquettes as energy sources. Today, biomass is increasingly used not only as an important indigenous energy source for production, but also as a commercial energy substitute for numerous users including consumer goods (Brkić and Janić, 2005, Martinov et al., 2011).
Following characteristics can be stated for this technology and its products:

1. Existence of huge source of renewable raw materials,
2. Average bulk density of briquettes range from 900 and 1100 kg/m3 and the possibility of a suitable packaging (foil, boxes, etc.) allows easy manipulation without contaminating the environment and the staff at work (Brkić and Janić, 2008),

3. Caloric value of briquettes, depending on sorts of raw material and moisture content, is ranging from 14,000 to 18,000 kJ/kg pointing to be similar to a good type of brown coal (Brkić and Janić, 2008),
4. Briquette combustion is very good, practically without smoke and no sulphur emissions, accompanied with low ash content, 2-3%,
5. Waste from the food industries (brewing spent grains, sediment sludge from oilseed processing, waste from the milling industry, withdrawn bread etc.) is an convenient raw material for briquette production for animal feed (Filipović et al., 1996, Filipovića et al., 1997, Filipović et al., 1995, Nježić et al., 2010).
6. In plants for separation of waste after separation, organic waste comprise over 60% in the morphological composition therefore it can be used in briquetting process (Nježić et al., 2006),
7. Existing combusting plants (various types of furnaces in households, smaller systems for heating buildings and manufacturing plants, etc.) enable the broadest application and use of briquettes as a source of heat,
8. Technological and technical concept simultaneously resolves: collection transportation, preparation, drying, briquetting and packaging of final products,
9. Market demand provide opportunities for practically unlimited amount of briquettes on both domestic and foreign markets and achieved sales prices provide a good cost-effectiveness of production and also a positive accumulation.

Due to the nutritional value of biomass consisting of: wheat, barley, oat, rye straw, maize, oilseed straw, sunflower stalks and remnants of the shell, waste from the milling industry, withdrawn bread, etc., it is necessary that the analysis of raw materials meet the requirements hygiene and health safety, microbiological and toxicological analysis by the Regulation on maximum permissible harmful substances, Off. SRJ 5/92, 11/92 and 32/2002, or meets the Regulation of the maximum amount of ingredients in animal feeds (sl. list SFRJ No. 2/90 and 27/90). Then biomass is used to produce animal feed, and otherwise in the process of briquetting as fuel. Parameters are examined according to table 1.

Table 1. Content of mycotoxin and heavy metals (Regulation on maximum permissible harmful substances, 2002, Regulation of the maximum amount of ingredients in animal feeds, 1990)

mycotoxin content (ELISA)	µg/kg
Aflatoxins B1+G1+B2+G2	<3
Ochratoxin	<10
Zearalenon	<1
Content of heavy metals (AAS)	mg/kg
Pb	0,4
Cd	0,005
As	0,5
Hg	0,03

For briquette making the production facility in Vršac was used. Briquettes were made from cereal straw, pruning branches of fruit trees and wood processing waste. Prior briquetting, moisture content in these raw materials was determined according standard AOAC procedure (1984).

There is no uniform standard in Europe, it‘s established by the European EPC pallet center standards in the report CEN/TS14961, so our country uses German DIN 51731. In table 2 is shown acceptable limits for content of heavy metals and ash by a European CEN/TS14961 report and DIN 51731/DIN plus standard.

Table 2. Content of heavy metals, ash (CEN/TS14961, DIN 51731)

Content	mg/kg
Pb	<10
Cd	<0,5
As	<0,8
Hg	<0,05
Cu	<5
S	<0,08%
N	<0,3%
ash	<3%

RESULTS AND DISCUSSION

Production of briquettes as a technical-technological process that aims to compress bulky material, i.e. to increase his bulk density. Is the tool for briquettes making, due to physical and chemical processes that are occurring, particles are tightly linked in a compact aggregates – briquettes. To be able to convert biomass to form briquettes following conditions must be ensured:

- moisture content of raw material must range from 10 to 12%
- particle size must not be greater than 5 mm

To ensure above mentioned technological requirements in most cases biomass should be grinded (chopped) and dried. After these preparations, raw material is brought into briquetting press where the briquetting form is made and than packed in appropriate packaging. Briquettes are usually circular or rectangular in cross section, minimum length of 50 mm. Biomass briquettes can be packaged in cardboard or plastic packaging.

Briquetting line capacity is up to 1,0 t/h at a moisture content of raw material not greater than 10%.

Briquetting plant consists of three technological lines:

1. Line for raw material preparation
2. Line for drying
3. Line for briquetting

For all types of raw materials and their combinations the optimal parameters were determined regarding: moisture, grain size, temperature, shape and size of the briquettes.

1. In straw briquetting trial work has not been used extra drying because the straw had 16% moisture. Straw briquettes were rectangular, measuring 270x100x50 mm, weight 1737 g. Analysis of the quality of briquettes is in acceptable limits according toTable 2.
2. In briquetting twigs the we used dryer as starting humidity was 45%. We have reduced the moisture to 16%. Briquettes of twigs were cylindrical with a diameter of 90 mm and length of 220mm, weight 1470 g. Analysis of the quality of briquettes is in acceptable limits according to Table 2.
3. In briquetting chaff extra drying was not used because the humidity was 14%. Briquettes of chaff were rectangular, measuring 270x100x50 mm, weight 1573 g. Results of analysis of briquettes are given in Table 3.

Table 3. Content of heavy metals, ash

Content	mg/kg
Pb	<10
Cd	<0,1
As	<0,2
Hg	<0,05
Cu	2,75
S	<0,08%
N	1,25%
ash	9 %

Results of analysis of briquettes from the chaff showed that it has 9% ash, 1.25% nitrogen, which exceeds acceptable limits, therefore mixing was done with sawdust in order to improve quality and meet the allowable value. Both raw materials were reduced to 15% moisture, and are mixed in ratio 50% chaff and 50% sawdust.

4. Briquettes from 50% chaff and 50% sawdust. Results of analysis of briquettes are given in Table 4.

Table 4. Content of heavy metals, ash

Content	mg/kg
Pb	<10
Cd	<0,5
As	<0,8
Hg	<0,05
Cu	1,47
S	<0,08%
N	0,25%
ash	1,36%

This briquette meets all established criteria, as briquettes for export to the EU. Moisture in all briquettes obtained were below 10%.

CONCLUSION

Moisture and granulation of raw material are crucial parameters for processes of collecting, transportation and briquettes processing. The production of briquettes from biomass originated from various raw materials attributed with different moisture content and granulation is completely solved in the production facility with automatic process regulation in Vršac. These briquettes are intended for domestic market and the European Union while meeting high standards of bio briquette quality in European Union. The presence of heavy metals is extremely dangerous because they accumulate in the body and when critical limits are reached they threaten the health of the population. The use of herbicides based on heavy metals and their quantity must be strictly controlled. To reduce the amount of heavy metals in the finished product, raw materials should be mixed with other materials that do not have heavy metals. Thus, it is necessary to make appropriate recipes from different types of raw materials.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III046012 – “Study of modern biotechnological procedures in the production of animal feed in order to increase competitiveness, quality and safety of the feed”, financially supported by the Ministry of Education and Science, Republic of Serbia.

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CHANGES IN CHEMICAL PROPERTIES OF CARROT DURING OSMOTIC DEHYDRATION IN SUGAR BEET MOLASSES

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ABSTRACT: Osmotic dehydration is an alternative method for reducing postharvest quality losses of vegetables, and a common process for producing dried vegetables which can be directly consumed or used as an ingredient in bread, cakes, pastries and many products of other food industries. The objective of the present study was to investigate the effect of using sugar beet molasses as an osmotic agent in the dehydration of carrot and to evaluate chemical characteristics of osmotically dehydrated products as compared to the *in natura* vegetables. Sugar beet molasses is an excellent medium for osmotic dehydration, primarily due to the high dry matter (80%) and specific nutrient composition. The paper describes the effects of different concentrations of sugar beet molasses (80, 60 and 40%) and immersion times (1, 3 and 5h) on osmotic dehydration/impregnation of carrot cubes. Osmotic dehydration process was conducted at constant temperature of 55℃ and under atmospheric pressure. During the process the solution to carrot ratio was 8:1. Analyses of variance (ANOVA) was used in order to find significant effects of solution concentration and immersion time on water loss, solid gain, dry matter, carbohydrates and minerals content. It was found that, both process variables had statistically significant effects ($p< 0.05$) on all examined responses, with the exception of sodium content ($p<0.10$). Immersion time was found to be more significant factor affecting water loss, dry matter content, carbohydrates and minerals content, while the effect of concentration was more evident on solid gain and magnesium content (significant at $p<0.05$).
Key words: *osmotic dehydration, carrot, sugar beet molasses*

INTRODUCTION

It is known that fresh fruit and vegetables constitute an important source of valuable and essential nutrient substances such as vitamins, minerals, cellulose and others. Unfortunately, fresh fruit and vegetables cannot be stored for a long time. High moisture content (for fruit and vegetables often higher than 80%) and water activity are conducive to developing of germs and putrefactive bacteria, which are responsible for decaying processes. For this reason fruit and vegetables are processed to extend their shelf life *(Kowalski and Mierzwa, 2011).*

Drying, especially hot air drying, is one of the most often applied industrial methods of preserving post harvested fruit and vegetables. This process stabilizes and prolongs the shelf life of fruit and vegetables and enables their longer storage and further utilization. High temperature and long time drying often change the shape, color, taste, aroma and nutrient properties of fruit and vegetables *(Mujumdar and Law, 2010).* Besides, drying is also one of the most energy-intensive unit operations in food processing industry. It was stated that drying easily accounts for up to 15 % of all industrial energy usage *(Chua et al., 2001; Kudra, 2004).* Therefore, alternative methods of drying or dehydration of products before drying are sought to minimize energy consumption and to improve the final product quality *(Min et al., 2010)*. The osmotic dehydration (OD) is one of these methods *(Shi & Maguer, 2010).*

Osmotic dehydration is a water removal process, based on soaking foods (fruit, vegetable, meat and fish) in a hypertonic solution. Water removal in liquid form, usage of mild temperatures and osmotic solution reusing are main advantages of osmotic dehydration process in comparison with other drying treatments *(Madamba, 2003).*

Osmotic dehydration as a pretreatment to many processes improves nutritional, sensorial and functional properties of food without changing its integrity *(Matusek and Merész, 2003).* In this way even 50 % of water, initially present, in the material can be removed at a relatively low expenditure of energy *(Kowalski and Mierzwa, 2011).* The literature contains earlier findings that state *(Lewicki and Lenart, 2006)* convective drying needs about 5 MJ per kilogram of evaporated water, compared to 0.1-2.4 MJ per kilogram of water removed in osmotic dehydration. The type of osmotic solution also plays an important role in the process, with salt solutions used for vegetables and sugar solution and syrups used for fruits *(Lewicki and Lenart, 2006*). Sugar beet molasses, as a by-product of sugar refining, is cheap and excellent medium for osmotic dehydration, primarily due to the high dry matter (80%) and specific nutrient content. It is also a significant source of numerous micronutrients (vitamins and minerals), especially K, Ca, Na and Mg *(Šušić and Sinobad, 1989)*. In this study, the effect of nutrients, present in the sugar beet molasses on the quality of osmodehydrated carrot was analysed.

MATERIAL AND METHODS

Carrot samples were purchased in a local market in Novi Sad (Serbia) and stored at 4º*C* until use. Initial moisture content, $X_{o,}$ was 89.65 ± 0.48 %. As osmotic agent, pure sugar beet molasses (around 80% solid content) and sugar beet molasses solutions (with 40% and 60% solid content) were used. Solutions were made by mixing pure molasses with distilled water. Sugar beet molasses was obtained from sugar factory in Pećinci, Serbia. Initial dry matter content in sugar beet molasses was 83.68%.

Carrots were washed thoroughly and peeled manually (using a stainless kitchen peeler). The peeled carrots were manually sliced into cubes, dimension 1x1x1 cm using a kitchen slicer. The amount of 100 g of sliced carrot cubes samples were prepared for each treatment. In all experiments, a weight ratio of solution to carrot samples of 8:1 (w/w) was used. The experiments were conducted under atmospheric pressure at solution temperature of 55º*C,* in an apparatus for osmotic dehydration, installed in the laboratory at the Faculty of Technology, Novi Sad.

The immersion lasted for 1, 3 and 5 hours. After osmotic dehydration, the samples were washed with water and gently blotted to remove excessive water from the surface. The next step was measuring the mass and determining of dry matter content, content of sucrose (S), total reducing sugar (TRS), invert sugar (IS) and some minerals (K, Na, Ca and Mg) in the fresh and dehydrated samples. The samples were kept in an oven (Instrumentaria Sutjeska, Serbia) at 105 ℃ for 24h, until constant weight was attained. Solid content of osmotic solutions was determined refractometrically *(Milić et al., 1992).* All analytical measurements were carried out in accordance to *AOAC (2000).*

Evaluation of mass exchange between the solution and the sample during osmotic dehydration was made by using the parameters such as final dry matter content (*DM)*, water loss (*WL)* and solid gain (*SG)* according to the equations presented in *Eren and Kaymak-Ertekin, 2006.*

Descriptive statistical analyses for calculating the means and the standard error of the mean, analysis of variance (ANOVA) and Tukey's post HOC tests, and Responce surface method (RSM) were performed using *StatSoft Statistica,* for *Windows, ver. 10* program. All obtained results were expressed as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

The osmotic process was studied in terms of WL, SG and DM content. An initial high rate of water removal (and solid uptake) followed by slower removal (and uptake) in the latter stages was observed. Rapid loss of water (and solid gain) in the beginning is apparently due to the large osmotic driving force between the fresh carrot cubes and the surrounding hypertonic

medium (sugar beet molasses). Figure 1 shows WL, SG and final DM content with respective standard deviation of experimental data, at the different osmotic dehydration conditions.

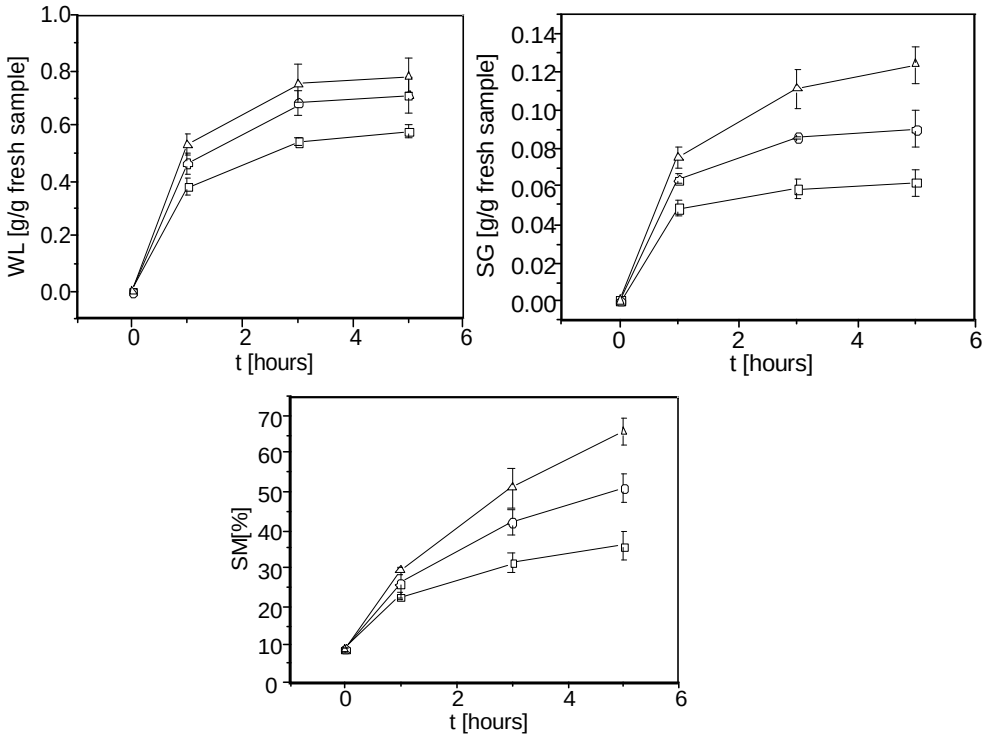


Figure 1. Changes in WL, SG and DM, during osmotic dehydration of carrot in different concentrations (40% □, 60% ○, and 80% △) of sugar beet molasses solutions

The initial values of WL and SG were set to zero, and the initial dry material content was 9.16±0.42%. The highest enlargement of WL (0.78 $g/g_{fresh\ sample}$), SG(0.12 $g/g_{fresh\ sample}$) and DM (65.82%) contents were observed after 5 hours of osmotic treatment in 80% sugar beet molasses concentration. Approximate values of corresponding parameters were obtained after 3h in 80% molasses solution (WL was 0,76 $g/g_{fresh\ sample}$, SG was 0,11 $g/g_{fresh\ sample}$ a DM was 51,1%). In the last stage of the process (last two hours) upward trend of kinetics parameters values is very small so it would be desirable to shorten the process to 3h and use 80% molasses solution, from economically aspect, considering the possibility of process expences reduction, and decreasing the energy consumption for heating.

The independent variables were osmotic time (X_1) of 1, 3 and 5h and molasses concentrations (X_2) of 40, 60 and 80% (by weight). The dependent variables observed were the response: water loss, *WL* (Y_1), solid gain *SG* (Y_2) and dry matter, *DM* (Y_3).The following second order polynomial (SOP) model was fitted to the data (*Montgomery, 1984*):

$$Y_k = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{2} \beta_{ij} X_i X_j, \quad (1)$$

where: β_{kn} are constant regression coefficients; Y, either DM (Y_1), WL (Y_2), or SG (Y_3); X_1 treatment time and X_2, solution concentration.

The ANOVA for kinetic terms (Table 1) reveals the significant effects of independent variables, dehydration time and sugar beet molasses concentration to the kinetics terms (WL, SG and DM). The SOP models for all variables were found to be statistically significant and the response surfaces were fitted to these models. The linear terms of SOP model were found significant, at 99% confidence level, and their influence were found most important in model calculation. DM was significantly affected by all process variables, at 99% confidence level. WL was also significantly affected by all process variables, at the same confidence

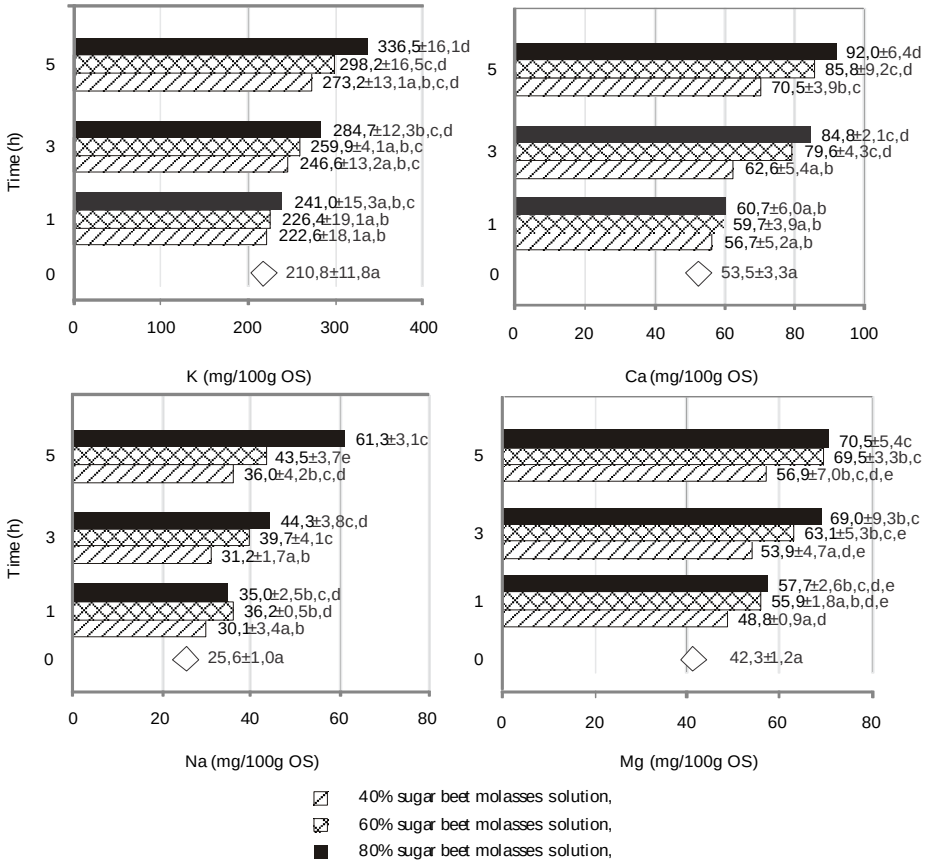
level. Each of these kinetics terms was most affected by treatment time. SG was significantly affected by treatment time and molasses concentration, but SG showed more sensitivity to the changes in molasses concentration. Table 1 shows that all kinetics SOP models had insignificant lack of fit tests, which mean that all the models represented the data satisfactorily.

Table 1. ANOVA for kinetic terms in carrots during osmotic dehydration in sugar beet molasses

Term	Factor	df	Sum of squares		
			WL	SG	DM
Linear	Time	1	5.25E-02^{+}	4.66E-04^{+}	398.489^{+}
	Concentration	1	2.13E-02^{+}	8.33E-04^{+}	146.511^{+}
Quadratic	Time	1	1.42E-02^{+}	1.19E-04*	18.148^{+}
	Concentration	1	1.16E-03^{ns}	2.00E-08^{ns}	0.394^{ns}
Cross-product	Time x Conc.	1	6.68E-04^{ns}	3.01E-04*	134.885^{+}
Lack of fit	Error	3	8.08E-04	2.75E-05	1.187
	r^2		98.582	98.544	99.927

$^{+}$Significant at 99% confidence level. *Significant at 95% confidence level.
**Significant at 90% confidence level. ^{ns}Not significant

Figure 2 represents changes in content of examined minerals, during osmotic dehydration in different concentration of sugar beet molasses solutions. The initial values of K, Ca, Na and Mg contents were 210.8±11.8 mg/100g, 53.5±3.3 mg/100g, 25.6±1.0 mg/100g and 42.3±1.2 mg/100g, respectively.



^{a,b,c,d,e} different letters printed within the same raw show significantly different means of observed data ($p<0.05$).

Figure 2. Changes in minerals content in carrot, during osmotic dehydration in sugar bet molasses solutions (mineral content expressed per 100 g sample).

The highest increase of mineral content was observed after 5 hours of osmotic treatment in 80% sugar beet molasses concentration (K was 336.5±16.1 mg/100g, Ca was 92.0±6.4

mg/100g, Na 61.3±3.1 mg/100g, and Mg 70.5±5.4 mg/100g). A statistically significant increase ($p < 0.05$) in content of the studied minerals, occurred after 3h of dehydration in 80% molasses solution. From the economic aspect it is cost effective to shorten the process duration to 3h during which the content of K, Ca, Na, and Mg increased to 284.7 ±12.3mg/100g, 84.8 ±2.1mg/100g, 44.3 ±3.8mg/100gand 69.0±9.3mg/100g and, respectively.

The SOP model (Eq. 1) was used to fit the minerals content data (K, Ca, Na and Mg). The SOP models (Table 2) for all variables were found to be statistically significant and the response surfaces were fitted to these models. The linear terms of osmotic time were found significant, at 95% confidence level, and their influence were found most important in model calculation. The sugar beet molasses concentration affects Mg content at 95% confidence level, while Na content is affected at 90% level of confidence. All mineral content in SOP models had insignificant lack of fit tests, which means that all the models represented the data satisfactorily.

Table 2. ANOVA for mineral content in carrots during osmotic dehydration in sugar beet molasses

Term	Factor	df	Sum of squares			
			K	Na	Ca	Mg
Linear	Time	1	2721.240*	1228.424*	514.089*	299.510*
	Concontration	1	252.630^{ns}	52.528**	22.130^{ns}	218.853*
Quadratic	Time	1	19.799^{ns}	39.644**	25.850^{ns}	15.357^{ns}
	Concentration	1	51.742^{ns}	3.144^{ns}	92.485^{ns}	37.706^{ns}
Cross-product	Time x Conc.	1	626.601^{ns}	10.167^{ns}	377.195*	162.601*
Lack of fit	Error	3	491.610	15.702	70.251	22.694
	r^2		94.537	99.235	97.423	98.364

$^{+}$Significant at 99% confidence level. *Significant at 95% confidence level.
**Significant at 90% confidence level. ^{ns}Not significant

The initial values of TRS, S and IS contents were 5.8±0.5g/100g, 4.0±0.2g/100g and 1.6±0.2 g/100g, respectively. The greatest increase was observed after 5 hours of osmotic treatment in 80% sugar beet molasses concentration (TRS 21.8±0.3 mg/100g, S 19.0±2.6 mg/100g, and IS 3.4±0.3 mg/100g). Since the aim of dehydration was not candying of fruits, it was necessary to minimize the penetration of sugar components in the samples of carrot. In this sense it is desirable to shorten the duration of the process at 3 h, whereas the content of S, IS, and TRS increased to 15.8 ± 0.8, 3.2 ± 0.1 and 17.0± 1.9 g/100g, respectively.

Table 3. Changes in carbohydrates content during osmotic dehydration of carrots in sugar beet molasses

	40%				60%			80%		
	0h	1h	3h	5h	1h	3h	5h	1h	3h	5h
TRS	5.8± 0.5^{b}	10.1± 0.7^{e}	14.5± 1.1$^{a.c}$	18.9± 0.9$^{d.f}$	12.0± 1.1$^{d.e}$	16.3± 0.3$^{a.d}$	20.2± 0.2$^{f.g}$	14.5± 1.4$^{a.c}$	17.0± 1.9$^{a.d}$	21.8± 0.3^{g}
S	4.0± 0.2^{a}	7.4± 0.9^{b}	10. 2± 0.7$^{b.c.d}$	14.0± 0.7$^{e.f}$	8.4± 0.6$^{b.c}$	12.1± 1.1$^{d.e}$	17.3± 0.9$^{g.h}$	10.8± 0.9$^{c.d}$	15.8± 0.8$^{f.g}$	19.0± 2.6^{h}
IS	1.6± 0.2^{b}	2.3± 0.2^{c}	2.9± 0.1$^{a.d.e}$	3.2± 0.2^{a}	2.5± 0.2$^{c.d}$	3.1± 0.1$^{a.e}$	3.3± 0.3^{a}	2.6± 0.1$^{c.d.e}$	3.2± 0.1^{a}	3.4± 0.3^{a}

$^{a.b.c.d.e.f.g.h}$ Different letters printed in superscript. within the same raw in the table. show significantly different means of observed data ($p<0.05$).

The SOP model (Eq. 1) was used to fit TRS, S, IS content data (Table 3). The SOP models for all variables were found to be statistically significant and the response surfaces were fitted to these models.

Table 4. ANOVA for sugar content in carrots during osmotic dehydration in sugar beet molasses

Term	Factor	df	Sum of squares		
			TRS	S	IS
Linear	Time	1	79.081^{+}	61.391^{+}	0.875^{+}
	Concentration	1	4.378*	6.748*	0.050^{+}
Quadratic	Time	1	0.247^{ns}	0.036^{ns}	0.094^{+}
	Concentration	1	0.008^{ns}	0.112^{ns}	0.003^{ns}
Cross-product	Time x Conc.	1	0.576^{ns}	0.663^{ns}	0.001^{ns}
Lack of fit	Error	3	0.651	1.874	0.003
	r^2		99.444	98.557	99.770

$^{+}$Significant at 99% confidence level, *Significant at 95% confidence level,**Significant at 90% confidence level, ^{ns} Not significant

The linear terms of osmotic time were found significant, at 99% confidence level, and its influence was found most important in model calculation. The sugar beet molasses concentration affects TRS and S content at 95% confidence level, while IS content is affected at 99% level of confidence. All sugars content SOP models had insignificant lack of fit tests, which means that all the models represented the data satisfactorily

ACKNOWLEDGMENT
This paper is a result of the research within the project III046005, TR – 31055, financed by the Ministry of Science and Technological Development, Republic of Serbia.

CONCLUSION

Both investigated parameters (immersion time, concentration) had statistically significant influence on WL, SG, DM, minerals and carbohydrates content, during osmotic dehydration of carrot cubes in sugar beet molasses. The ANOVA and RSM were successfully used for determination of the system responses, all input variables were found statistically significant while predicted and observed responses correspond very well. Process time, in comparison with solutions concentration, had more statistically significant effect on all tested variables, with exception of SG. Increase of valuable bioactive components in dehydrated carrots contributes to improving theirs nutritional quality. Some increase in sugar content, combined with reduced moisture content, contributing to sustainability of this semi-final product. According to the results, it can be concluded that proposed process conditions for improving product quality were found to be: osmotic time of 3h and sugar beet molasses concentration of 80%. Considering the shorter immersion time and thus lower energy consumption in heating system, the proposed conditions are also acceptable from economic aspects of production.

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CHARACTERISTICS OF SPELTA WHEAT AS A RAW MATERAL FOR ORGANIC PASTA

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ABSTRACT: In the past few decades there has been a cosiderable intrest in consumption of ancient wheat. Spelta wheat is growing without the use of pesticides in harsh ecological conditions and marginal areas of cultivation. It is believed that primitive wheat species are more disease-resistant and can produce healthier foods than those made from modern varieties. Spelta wheat is suitable raw material for production organic food like of pasta, specialty type of bread and other products of altered nutritional characteristics compared to conventional wheat products. In determining the purity of products spelta aleuron layer plays a key role. This paper investigates the technological quality and dough rheology of wholemeal spelta flour as a raw material for pasta. Technological quality of wholemeal spelta is defined by basic chemical analyses (protein, starch, fat, cellulose, ash and reducing sugars) and metal content of Ca, Zn, Cu, Mn, and Fe. Rheology of wholemeal spelta is defined by gluten characteristics, farinogram and alveogram. Activity of amylases is defined by Falling number and amylograph. Beside chemical and rheological properties the convenience of spelta wholemeal flour for pasta is evaluated on the data concerning cooked pasta quality. Data point at good indicators of technological quality and sensor characteristics of spelta pasta. Pasta obtained from wholemeal spelta flour may be a new organic safe product at the market.
Key words: *wholemeal spelta flour, quality of spelta pasta, pasta color*

INTRODUCTION

Spelta wheat (*Triticum aestivum* subsp. *spelta*) is an old European crop. In the past few decades there has been a cosiderable intrest in consumption of ancient wheat. Spelta wheat is growing without the use of pesticides in harsh ecological conditions and marginal areas of cultivation. It is believed that primitive wheat species are more disease-resristant and can produce healthier foods than those made from modern varieties (Bonafaccia at all 2000, Abdel-Aal & Rabalski 2008).

The nutritive value of spelta wheat is high and contains all the basic components which are necessary for human beings such as starch and nonstarch carbohydrates, proteins, lipids, vitamins and minerals. Spelta wheat is reported to have higher protein content (16-17%) than *Tr. Aestivum* due to higher protein of aleuron layer (Bojanski and Frančakova, 2002). Spelta varieties have shown potential in various food applications, including bread, pasta, breakfast cereal and other products of altered nutritional characteristics compared to conventional wheat products. With so much interest in organic foods, organically-grown spelta finds its way to the food market (Marconi at all 2002, Abdel-Aal & Rabalski 2008).

This paper investigates the technological quality and dough rheology of wholemeal spelta flour as a raw material for pasta. Technological quality of wholemeal spelta is defined by basic chemical analyses (protein, starch, fat, fiber, ash, moisture and reducing sugars) and metal content of Ca, Zn, Cu, Mn, Fe. Rheology of wholemeal spelta is defined by gluten characteristics, farinogram and alveogram. Activity of amylases is defined by Falling number and amylograph. Beside chemical and rheological properties the convenience of spelta wholemeal flour for pasta is evaluated on the data concerning cooked pasta quality

MATERIALS AND METHODS

Wholemeal flour of two spelta varieties (Nirvana and Eco 10) is tested.
Basic chemical analyses (protein, starch, fat, cellulose, ash) of wholemeal spelta flour are defined according to the Regulations on the physical and chemical methods of analysis for quality control of grain, milling and bakery products, pasta and deep frozen dough (1988),
Falling number according to ICC Standard No 107/1, gluten index and gluten index after 1h at 30 ℃ according to ICC Standard No 155 , reducing sugars content according to Kaluđerski and Filipović 1998 and metal content (Ca, Zn, Cu, Mn and Fe) ,according to methods practiced in accredited FINS Laboratory 2011. Rheology of wholemeal spelta is defined by farinogram *(*Kaluđerski and Filipović, 1998*)* and alveogram (AACC, 1994*)*
Pasta is made on the device "La Parmigiana D45" MAC 60, LaParmigana Italy. Moisture content of the dough is adjusted to 31,5%, mixing time was 15 min. Quality of cooked pasta was determined on the behaviour of pasta during cooking according to the method described by Kaluđerski and Filipović (1998).

RESULTS AND DISCUSSION

Quality of wholemeal Spelta flour of two different varieties is presented in Table 1. Moisture and ash content of both wholemeal varieties are fairly consistent, but the ash content is significantly higher than the ash content of flour from wholemeal vulgare wheat (Kaluđerski, Filipović 1998). The high ash content is a consequence of the characteristics of spelta grain anatomy, i.e. high ratio of bran layer, deep crease, lower endosperm content and different distribution of minerals within the grain in comparison to bread wheat grains and the statement is consistent with data of Ruibal-Mendieta et al., (2005). Investigated spelta varieties are characterized by high protein content (Table 1) which qualifies this raw material as a suitable for pasta production. In addition to the high total protein content, gluten proteins are attributed with good quality. Gluten index (Table 1) was significantly lower in Eco variety in relation to the Nirvana, indicating increased activity of proteolytic enzymes which is confirmed by extremely low value of gluten index after 1h. Regarding variety Nirvana an increased activity of proteolytic enzymes is indicated by gluten index after 1h. The content of starch, fat and fiber have are relatively uniform in both varieties. It is worth emphasizing that spelta contains more fiber than the bread wheat varieties in which the average value is round 2.0% (Vukobratovic, Dozet 1992). Data concerning content of individual elements vary depending on the variety with the greatest difference in Fe content. The high mineral content experienced in spelta varieties point at the improved bioavailability of those elements, due to low phytic acid content reported by and Ruibal-Mendieta et al. (2005).

Table 1. Parameters of quality of wholemeal spelta flour

Parameters of quality	Nirvana	Eco 10
Moisture content (%)	13.25	12.82
Ash content (% d.m.)	2.36	2.37
Crude protein content (% d.m.)	18.17	16.02
Cellulose content (% d.m.)	2.29	2.16
Starch content (% d.m.)	57.59	57.3
Fat content (% d.m.)	2.20	2.23
Gluten index	95	68
Gluten index (after 1h at 30°)	57	50
Ca content (mg/kg)	245.38	299.95
Zn content (mg/kg)	30.0	33.38
Cu content (mg/kg)	4.89	3.98
Mn content (mg/kg)	43.76	46.45
Fe content (mg/kg)	41.19	37.9

Based on farinograph data. (Table 2) water absorption. dough development and stability are quite uniform and can be considered optimal for bread making but not for making pasta. A variety of eco 10 has an extremely high 15 min drop indicating a high proteolytic enzyme activity and thus classified as a quality class C1. Alveograph data: tenacity, length of curve, dough extensibility and baking strength depend on the variety characteristics, while the value of P/L is even (Table 2).

Table 2. Rheology of wholemeal spleta flour

Farinogram	Nirvana	Eco 10
Water absorption (%)	63	64.1
Dough development (min)	3.5	3
Dough stability (min)	0.5	0.5
15 min drop (FU)	80	150
Number of quality /quality class	59.4 / B 1	41.2 / C 1
Alveogram		
Tenacity (mm)	75	55
Length of curve (mm)	25	18
Dough extensibility G (ml)	11.1	9.4
Baking strength W (10^{-1}J/kg)	74	40
Configuration ratio P/L	3.0	3.06

A slightly higher content of reducing sugars is experienced with Eco 10 than with Nirvana variety. Both values are considerably higher than that average values for vulgare wheat (Vukobratovic and Dozet 1992) (Table 3). Falling number and amylograph data of Nirvana are within the optimum range for baking contrary to Eco 10 where they indicate an increased activity of alfa amylase (Table 3). Concerning the quality for pasta, both varieties could be considered as inconvenient due to increased amylase activity.

Table 3.. Activity amylolitic enzymes of wholemeal spelt

Characteristics	Nirvana	Eco 10
Falling number (s)	261	225
Content of reducing sugars (% d.m.)	4.15	4.62
Amilograph maximum viscosity (AU)	370	120

Table 4. shows that pasta Eco 10 is attributed with significantly higher percentage of water absorption compared to the Nirvana pasta, resulting in an increased volume of cooked pasta. Increased cooked pasta volume indicates the possibility of starch swelling and this property is in both cases in the optimal range. Higher residue in cooking water is attributing Eko 10 pasta compared to Nirvana, probably due to lower protein content and high activity of proteolytic enzymes (Table 1). In addition to above pasta features. for consumers is a very important impression when chewing. Properly cooked pasta should provide resistance to the bite and not to give the impression of creamy products. Eco 10 pasta is attributed with high stickiness contrary to Nirvana pasta (Table 4). On the whole, pasta made of wholemeal spelta has good sensor characteristics and much improved nutritive characteristics in comparison to commercial pasta that is at our market.

Table 4. Quality characteristics of spelt pasta

Quality of pasta cooking	Nirvana	Eco 10
Water absorption (ml/100g)	144.0	153.5
Cooked pasta volume increase (α)	2.8	2.9
Residue in cooking water (% d.m..)	6.8	7.4
Stickiness (max -0. min 10)	8	5

CONCLUSIONS

Based on the investigation concerning convenience of spelt varieties Nirvana and Eco 10 for whole meal pasta it can be concluded:

- The protein and gluten content of both spelt varieties reach values greater than the values commonly achived for bread wheat and these values approache the values of durum wheat protein and gluten content.
- Rheology data point at increased activity of proteolytic and amylolitic enzymes, particularly for Eco 10.
- Beside the unfavorable rheological properties spelta wholemeal flour proved to be convenient for pasta.
- Data of spelta pasta quality point at good technological quality and sensor characteristics of spelta pasta. Pasta obtained from wholemeal spelta flour may be a new organic safe product on the market.

ACKNOWLEDGEMENTS

These results are part of the project supported by the Ministry of Education and Science of the Republic of Serbia. TRI 46005.

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CONVECTIVE DRYING KINETICS OF RASPBERRY

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ABSTRACT: The drying kinetics of raspberry in a laboratory dryer was studied. Fresh, untreated raspberries were dried at selected temperatures of 50 ^{o}C with a constant air velocity of 1.2 m/s. The drying rate curves showed that drying process took place only in the falling rate period. Thin-layer drying models of Newton-Lewis, Henderson and Pabis, logarithmic, Page, Wang and Singh evaluated based on coefficient of determination (R^2), reduced chi-square (χ^2) and root means error (RMSE). The logarithmic model was found to be the best model for describing the characteristics of raspberry. The changes of color on raspberry's surface were determined by original approach, combining digital camera, computer and graphic software. Pearson correlation coefficients and triplicate sample paired t-test on R (red) colour index frequency were calculated during the experiment. A mathematical model to predict the shrinking of geometrical bodies was proposed, assuming unidirectional drying and two-dimensional shrinkage. The model was numerically solved by finite differences, taking into account a convective term in the mass balance equation, which appears as a consequence of non-unidirectional shrinkage. Thermal analysis, by means of differential scanning calorimetry (DSC) and thermogravimetry (TGA) of fresh and dried raspberries have been studied. From results obtained differences in thermal stability of fresh and dried raspberries were shown.
Keywords: *Raspberry; Convective drying; Kinetics; Digital image analysis; Shrinking; Thermal analysis*

INTRODUCTION

Drying of moist materials is a complicated process which involving simultaneous, combined heat and mass transfer phenomena, which occur in the material being dried (Yilbas, et. al 2003). One of the most widely used methods of food (fruit and vegetable) preservation is convective drying process, which finds its principal purpose in removing of water in the solid material, up to certain level, at which microbial spoilage and deterioration chemical reaction are greatly minimized (Krokida, et. al 2003). The quality of products is considerably improved, by understanding the heat and mass transfer in the product, and also the drying process parameters. Drying process activity is influenced by a number of internal parameters, which characterize the material, such as density, permeability, porosity, sorption-desorption characteristics and their physical properties, and also external parameters, related to drying process, like drying temperature, drying air velocity, sample dimensions and relative humidity of the drying medium). In most drying processes water is removed by convective evaporation in which heat is supplied by hot air.
Mathematical modeling is widely used in developing kinetics models to predict the mass transfer of drying process at atmospheric pressure. However, it is very difficult to develop a mathematical model capable to incorporate all above mentioned factors (both internal and external) involved in the process (Kaya, et. al, 2007, Lewicki et. al., 2004, Sacilik, et. al. 2006, Srikiatden, et. al. 2005, Uretir, et. al. 1996, Velić, et. al. 2004).
The main objective of this study was to determine the effects of process parameters like drying air temperature, air velocity and sample surface on the diffusion model coefficients and the values of moisture diffusivity and heat transfer coefficients by using multiple

regression analysis, and to test the most appropriate drying model for understanding the drying behavior of raspberry.

MATERIALS AND METHODS

The laboratory convective dryer, consisted of an electric 5000 W heater placed inside the heating chamber. In the measurements of temperatures, J type iron–constantan thermocouples were used with a manually controlled 10-channel automatic digital thermometer (Omega, MDS Si8-TC-C4EI-AL, USA), with reading accuracy of ±0.1 ^{o}C. A thermo hygrometer (Vaisala HMT330) was used to measure humidity at various locations of the system. Moisture loss was recorded at approximately 5 min intervals during drying process for determination of drying curves by a digital balance (Napco, JA3000S, China), with measurement range of 0–3000 g and an accuracy of ±0.01 g. The raspberries were thoroughly washed before drying process. Samples were selected from the lot for uniformity using the specific gravity of the fruits.The tray was loaded as thin layer, and the initial weight of the raspberries on each tray was approximately 35 g. The raspberries (aver. diameter 11.4 mm, and aver. weight 2.9 g for single fruit), were carefully and orderly placed on the trays, which were not perforated. The initial and final moisture contents of the products were determined at 105 ^{o}C by using an Infrared Moisture Analyzer (METTLER, LJ16, Switzerland). After the dryer is reached at steady state conditions for operation temperatures, the samples are put on the tray of dryer and dried there During the experimental drying, raspberries of average initial moisture content 85.7% (wb), were dried, at temperatures of 50^{o}C, relative air humidity 50-55%, in atmospheric convective dryer, at the velocities of drying air of 1.2 m/s. The final moisture contents represent moisture equilibrium between the sample and drying air under dryer conditions, beyond which any changes in the mass of sample could not occur.

Color images of raspberries during drying process were captured in triplicate, by a Canon PowerShot A550 CCD camera, which is a common digital cammera for home use. All the acquired images were 24 bit RGB (16.8 millions of colors) with a 1024 x 768 spatial resolution. The macro function of the digital camera has been used, to cover a scene area of approximately Ø10 cm. Each sample was chosen to be at the approximately constant thickness of 5 mm and to an area sufficient to cover completely the image scene, in order to avoid the presence of background in the image. Then, it was placed on a white paper napkin set on a flat white painted surface, 15 cm below the digital camera. Paper napkins were used in order to absorb the excess of oil and therefore to avoid undesired reflection effects. With this setup, it was possible to capture images with negligible shadows and without specular reflections. The acquired RGB images were transferred to a computer in the form of .jpeg compressed image files. The size of the imported jpeg images ranged from 900 to 1250 KB for the 9 x 22 = 198 images of the data set. For the elaboration of the images, every image file is imported in the originally developed computer program.

The dimensions of raspberries were measured by a digital micrometer.

The kinetics models were selected with the correlation coefficient (R^2)., the reduced chi-square (χ^2), mean bias error (MBE) and root mean square error (RMSE) were used to determine the quality of that fitting function (Akpinar, 2006; Midilli et al., 2002, Mujumdar, et. al., 1995).

These commonly used parameters can be calculated as follows:

$$\chi^2 = \frac{\sum_{i=1}^{N}(MR_{\exp,i} - MR_{pre,i})^2}{N-n},\ RMSE = \left[\frac{1}{N}\cdot\sum_{i=1}^{N}(MR_{pre,i} - MR_{\exp,i})^2\right]^{1/2},$$

$$MBE = \frac{1}{N}\cdot\sum_{i=1}^{N}(MR_{pre,i} - MR_{\exp,i}) \quad (1)$$

where $x_{exp,i}$ stands for the experimental values and $x_{pre,i}$ are the predicted values by calculating from the model for these measurements. N and n are the number of observations

and constants, respectively. For quality fit, R^2 value should be higher, and χ^2, MBE, RMSE and values should be lower (Yaldiz, et. al 2001; Ertekin, et. al 2004).
Analysis of variance (ANOVA) were performed using StatSoft Statistica, for Windows, ver. 10 program. The model was obtained for each dependent variable where factors were rejected when their significance level was less than 95%.
Differential scanning calorimetry (DSC) and thermogravimetry (TGA) of fresh and dried raspberries have been performed on TA Instruments DSC Q 1000, differential scanning calorimeter and TGA measurements on TA Instruments TGA Q 500 thermogravimetric analyzer under N_2 purge flow of 50ml/min and 60ml/min respectively. DSC scans were conducted in temperature range from -90ºC to 400ºC, with heating rate Hr=5Cº/min, and TGA scans were performed in temperature range of 25ºC to 900ºC with heating rate Hr=5Cº/min.

RESULTS AND DISCUSSION

Kinetics study

The moisture content data at the given drying conditions fitting computations with the drying time were carried on the several drying models evaluated by the previous workers (see Table 1). The moisture ratio (MR), as well as the R color index and overall fruit diameter changes during the drying experiment are presented on Fig. 1.

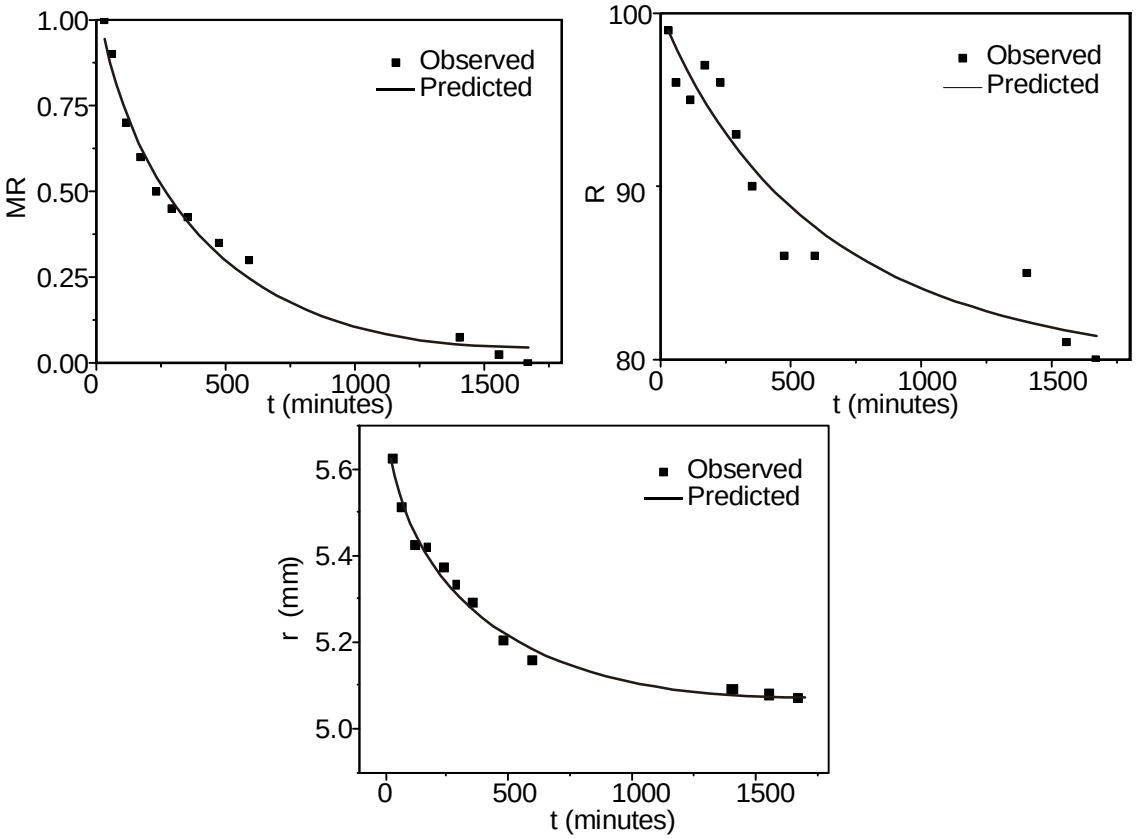

Figure 1.Raspberry moisture ratio MR, R color index and fruit radius during drying process at temperature of 50 °C, atmospheric pressure, 1.2 m/s air flow velocity

The statistical analyses results applied to the common mathematical models of drying process at 50°C air temperature and 1.2 m/s air velocity are given in Table 1. The best model describing the thin layer-drying characteristic was chosen as the one with the highest r-value and the lowest χ^2, MBE and RMSE values. From Table 1, it was determined that the

best fitting results were obtained with logarithmic model (estimated coefficients were: *a*=0.989; *k*=0.003; *c*=0.034).

Table 1. Thin layer drying curve models

Model name	Model	R^2	χ^2	MBE	RMSE
Newton-Lewis	$MR = \exp(-k \cdot t)$	0.918	8.94E-02	-1.82E-01	3.15E-01
Page	$MR = \exp(-k \cdot t^n)$	0.863	4.82E-02	-2.70E-02	4.68E-02
Henderson and Pabis	$MR = a \cdot \exp(-k \cdot t)$	0.924	2.63E-02	-1.06E-03	1.83E-03
Logarithmic	$MR = a \cdot \exp(-k \cdot t) + c$	0.937	2.22E-02	-1.33E-03	2.31E-03
Wang and Singh	$MR = 1 + a \cdot t + b \cdot t^2$	0.875	8.33E-02	-1.44E-01	2.50E-01

Table 2 shows the results of the statistical analyses results applied to the common mathematical models of color changes during the drying process. The best model describing the color changes characteristic was logarithmic model (coefficients were: *a*=19.402; *k*=0.002; *c*=80.494).

Table 2. Red color index changes models

Model name	Model	R^2	χ^2	MBE	RMSE
Newton-Lewis	$R = \exp(-k \cdot t)$	0.914	1.58E-02	-6.88E-02	1.19E-01
Page	$R = \exp(-k \cdot t^n)$	0.945	5.11E-03	4.09E-03	7.08E-03
Henderson and Pabis	$R = a \cdot \exp(-k \cdot t)$	0.912	7.96E-03	8.86E-05	1.53E-04
Logarithmic	$R = a \cdot \exp(-k \cdot t) + c$	0.958	3.93E-03	-3.36E-08	5.81E-08
Wang and Singh	$R = 1 + a \cdot t + b \cdot t^2$	0.951	4.64E-03	-8.25E-03	1.43E-02

For a mixture of components with constant specific volumes, the mass conservation equation can be written as (Viollaz, et. al. 2002),:

$$\frac{\partial \rho_A}{\partial t} = D \frac{\partial^2 \rho_A}{\partial^2 x} - v \frac{\partial \rho_A}{\partial x} \tag{2}$$

where: ρ_A is mass concentration of drying material (kg water/m^3 of total volume), t - time (s), D is mutual diffusion coefficient (m^2/s), x - coordinate, (m), v - volume average velocity in direction x (m).

Eqn. (2) will be solved numerically using the following assumptions: the partial specific volumes of water and dry solid are constant; moisture diffusivity is constant; drying is unidirectional; shrinkage is three-dimensional.

By expressing the change of volume with time as the product of velocity times area, the volume average velocity at the position x due to the change of the lateral area can be obtained (Viollaz, et. al. 2002):

$$v = -\frac{x}{A}\frac{dA}{dt} \tag{3}$$

Eqs. (2) and (3) were solved numerically using the following initial and boundary conditions:

$$t = 0; \quad \rho_A = \rho_{A0}; \quad x = 0; \quad \frac{\partial \rho_A}{\partial x} = 0; \quad x = L; \quad \rho_A = \rho_{A\infty} \tag{4}$$

Evalutive procedure starts with the average raspberry fruit radius of 5.6 mm. Diffusion coefficient for long drying periods, can be simplified to first term of the series, and moisture ratio can

be expressed as M/M_0, due to relatively small value of M_e compared to M or M_i, written in the logarithmic form:

$$\ln\frac{M}{M_e} = \ln\frac{6}{\pi^2} - \left(\pi^2 \cdot \frac{D_{eff} \cdot t}{r^2}\right), D_{eff} = D_0 \cdot \exp\left(-\frac{E_a}{T \cdot R}\right), \quad (5)$$

where the effect of temperature on effective diffusivity is expressed using Arrehnius type relationship. E_a is the activation energy of moisture diffusion (kJ mol^{-1}), D_0 is the diffusivity value for infinite moisture content, and R represent universal gas constant (kJ mol^{-1}). T is absolute drying air temperature (K). Calculated effective diffusion coefficient was 3.2E-10m^2/s, and activation energy of 20 to 40 kJ/mol, which were in accordance to literature values (Lewicki, et. al. 2004).

The obtained results for prediction of fruit radius corresponds very well to those achieved by experimental measurement. The obtained statistical parameters are, as follows: R^2=0.934; χ^2=1.78E-02; MBE=4.12E-03; RMSE=8.18E-03.

Thermal analysis

Obtained DSC and TGA curves of fresh and dried raspberries, (Fig 2.) show differences of thermal stability between fresh and dried fruits caused by water loss during drying process (Syamaladevi et. al, 2009). On Fig 2a DSC curves for fresh (dashed curve) and dried (solid curve) fruits are presented. From DSC curve for fresh raspberries it can be seen that phase trasition of ice melting and water evaporation are dominant thermal events, as the sample water content is high. High water content (85.7%) is also observed from corresponding TGA curve of fresh raspberries (solid curve Fig 2b). DSC curve of the dried sample, Fig 2a (dashed curve) is characterized by three edothermal peaks with maximums at T_{m1}=-40.7ºC, T_{m2}= -20.23 ºC and T_{m3}=114.0ºC respectively and one glass transition T_g= 42.3 ºC. Results are in agreement with Syamaladevi et. al, 2009, who showed that glass transition temperatures of raspberries are influenced by water content, and Shyam et al 2010, Kim et. al, 2001, as it was found that dried food material has similar thermal properties of semicrystalline polymers. Water content of dried sample obtained from TGA curve, Fig 2, dashed curve was 19.3%.

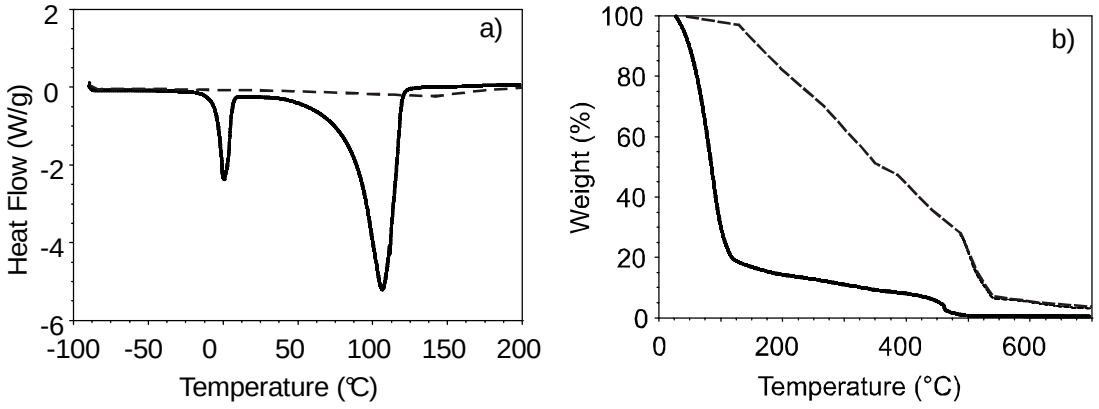

Figure 2. a) DSC curves of dried (dashed curve) and fresh (solid curve) raspberries and b) TGA curves of dried (dashed curve) and fresh (solid curve) raspberries

CONCLUSION

In order to explain the drying behavior and develop the mathematical modeling of raspberry convective drying process, several models, were applied, upon the changes in raspberry moisture ratio. The best model (logarithmic) adequately describe the drying behavior of raspberry fruit in the drying process, with gained r^2 of 0.937 and χ^2 of 2.22E-02.

Also, a few mathematical model of browning process during drying were developed and tested. Statistical analysis, including comparison tests, showed that logarithmic model adequately represent the browning process, showing r^2=0.958 and χ^2=3.93E-03.
Finally, a mathematical model to predict the shrinking of geometrical spherical bodies was proposed, assuming unidirectional drying and two-dimensional shrinkage. This model showed good results, also, with r^2=0.934 and χ^2=1.78E-02.
Thermal analysis shows differences of thermal stability between fresh and dried fruits caused by water loss during process of drying.

ACKNOWLEDGEMENT

The authors acknowledge for the financial support the Ministry of Science and Technological Development of the Republic of Serbia, TR – 31055, TR – 31093, 2011-2014

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DETECTION OF VOLATILE COMPOUNDS IN DOUGH DURING MIXING

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ABSTRACT: Dough mixing is very important stage in the bread making process. The mixing process promotes numerous physical, chemical and physic-chemical modifications that conduct to dough development. The aim of this work was to investigate the mixing time and temperature influence on volatile compounds in dough made from flour blend (triticale, hull-less barley, rye, rice, and corn flour). Dough was made from flour blend, sugar, salt, yeast and water, according to the recipe and technology used in wheat bread making. An investigation of volatile compounds was done using solid-phase microextraction (SPME) and gas-chromatography mass-spectrometry (GC-MS). Volatile compounds were analyzed during dough mixing after 6, 8, 10 and 15 minutes at dough temperature 20, 25 and 32 °C. The optimal parameters for qualitative dough making (appropriate elasticity) could be: time 6 min and temperature 32 °C, time – 8 and 10 min and temperature 25 °C. There were detected 13 volatile compounds in the mixing process. Identified volatile compounds belong to alcohols, aldehydes, carboxylic acids and terpens. Part of identified volatile compounds such as hexanal, D-limonene, 1-hexanol, 1-octen-3-ol and heptanol detected in dough originate from flour blend. Some compounds were released from dough due to physical and chemical changes taking place in the dough mixing process. Total peak area of volatile compounds increased extending dough mixing time and temperature.
Key words: *mixing, triticale, volatile compounds, solid-phase microextraction*.

INTRODUCTION

In production of bread and pastry various grinds of wheat and rye flour traditionally are used, though new possibilities for expanding of the assortment are sought. Researchers worldwide have been studying use of triticale, hull-less barley, maize and rice in bread and pastry production (Taketa *et al*., 2004; Ozola et al., 2011).
Triticale (*Triticosecale wittmack*) is the first man-made cereal produced by crossing wheat (*Triticum spp.*) and rye (*Secale ceral L.*). The nutritional value of triticale is close to that of wheat and rye, and although some triticale-based food can be purchased at health food stores. (Salmon et al., 2002; Hosseinian and Mazza, 2009). Triticale is mainly used as an ingredient in animal feeding, but also on a smaller scale as a food ingredient, for example in bread making or as replacement for soft wheat in biscuits, cakes and cookies (Sabovics et al., 2010; Rakha et al., 2011).
Bread making is basically a temperature-dependent two-step process, consisting of fermentation, in which CO_2 production associated with yeast activity is manifested in a dough volume expansion, and baking, in which yeast activity is terminated and the bread structure is finalized (Bajd and Serša, 2011).
The first step in a baking process is dough mixing. How the mixing is performed and the ingredients are incorporated and dispersed largely determine the quality of the baked product (Gras et al., 2000; Aamodt et al., 2003). The mixing process promotes numerous physical, chemical and physic-chemical modifications that conduct to the dough development (Aït Kaddour et al., 2008). Mixing contributes three main functions in structure formation of dough: homogenization, it blends the ingredients into a macroscopically homogeneous mass; air inclusion which will form nuclei for gas bubbles that grow during dough fermentation and gluten development via mechanical energy input (Peighambardoust et al., 2010; Koeler et al., 2010). In dough mixing, the distribution of dough ingredients and the hydration phase

occur concomitantly with the mechanical energy input (Peighambardoust et al., 2010). The dough must be mixed for a specific time identified as an optimum dough development to ensure optimal loaf volume and bread texture (Aït Kaddour et al., 2008).
The aim of this work was to investigate the influence of the mixing time and temperature on volatile compounds in dough made from triticale, hull-less barley, rye, rice and maize flour.

MATERIALS AND METHODS

Experiments were carried out at the Faculty of Food Technology, Latvia University of Agriculture. The data are reported as means of measurements made in triplicate, where each sample was obtained from a separately prepared batch of dough for each formulation.

Materials

Triticale, rye and hull-less barley crops of 2011 cultivated at the Priekuli Plant Breeding Institute (Latvia) were used in the current study. Triticale, rye and hull-less barley used for study were ground in a laboratory mill Hawos (Hawos Kornmühlen GmbH, Germany) obtaining whole grain fine flour. Rice and maize flour was purchased from Joint Stock Company Ustukiu Malunas (Lithuania). For this research there was used flour blend that contains 60% - whole grain triticale, 15% - whole grain hull-less barley, 15% - whole grain rye, 5% - rice and 5% - maize flour. Ingredients, such as sugar, salt, water and yeast are included in dough formulation in order to improve sensory properties and keep quality of bakery products.

Sample preparation

All flour samples were mixed together in one flour blend. For dough sample preparation there was used flour blend (250g), dried yeast (7.8 g), sugar (4.5 g), salt (3.8 g) and water (170 ml). Water temperature was calculated from formula (1) and it was adjusted accordingly before adding to flour blend. The preparation of samples and experiment scheme are shown in Figure 1.

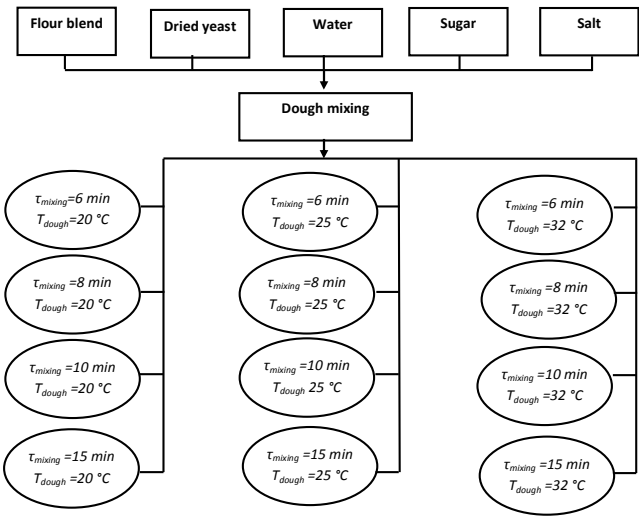

Figure 1. The preparation of samples and experiment scheme

The following abbreviations of the samples in the article are used:

- 6-20, 6-25, 6-32;
- 8-20, 8-25, 8-32;
- 10-20, 10-25, 10-32;
- 15-20, 15-25, 15-32.

Where the first number in the sample code: 6, 8, 10 and 15 – means dough mixing time in the experiment, but the second number 20, 25 and 32 – corresponds dough temperature.

Dough temperature
To obtain the required temperature of the dough the following calculation formula was used:

$$T_{water} = (2 \times T_{dough}) - T_{flour\ blend} \quad (1)$$

where:
T_{water} – temperature of the water, ºC;
T_{dough} – required dough temperature, ºC;
$T_{flour\ blend}$ – temperature of the flour blend, ºC.

Detection and identification of volatile aroma compounds
Volatile compounds were determined from flour blend dough samples using solid-phase microextraction (SPME) in a combination with gas chromatography/mass spectrometry. The SPME fibre was coated with a thin polymer film – Carboxen/Polydimethylsiloxane (CAR/PDMS). The film thickness is 85 μm with bipolar polarity (Supelco, Inc., USA). Volatile compounds were absorbed from dough to fibre in a closed mixer container in the mixing process at various temperatures for various duration according to the experimental design. Volatile compounds from fibre were thermally desorbed in the injector of a gas chromatograph-mass spectrometer '*Clarus 500 GC/MS'* (PerkinElmer, Inc., USA). The SPME extraction time was done for different duration of dough mixing: the first - extraction time is 6 min at dough temperature 20±2 °C without pre-incubation, the second – 6 min at 25±2 °C and the third – 6 min at 32±2 °C. Other samples were mixed for 8, 10 or 15 min at the above mentioned temperature.
Separation of volatiles was carried out in the Elite-Wax (PerkinElmer, Inc., USA) capillary column (60 m x 0.25 mm i.d., DF 0.25 μm). The details of the program used in GC–MS analysis are following: the initial temperature was 40 ºC, held for 7 min, then ramped from 40 °C to 160 °C at a rate of 6 °C min^{-1} and from 160 ºC to 210 °C at a rate of 10 °C min^{-1} with a hold time for 15 min. The total run time was 47 min for a sample. Mass spectrometer in Electron impact Ionization mode was set on 70 eV as the electron energies, while the ion source temperature was set to 250 °C and inlet line temperature was set to 250 °C. Injections were performed in splitless mode and helium (He) was used as carrier gas at a constant flow of 1 ml min^{-1}. Acquisition parameters in full scan mode: scanned m/z 40-300. Compounds were identified by comparison of their mass spectra with mass spectral library Nist98.

RESULTS AND DISCUSSION

It is an important factor in breadmaking that dough temperature should be kept under control during dough mixing (Li and Walker, 1992). To make dough in mixing process with required temperature (20±2, 25±2 and 32±2 °C) water temperature should be around 14, 21 and 42 °C, respectively. Temperatures of added water and mixed dough are shown in Table 1.
The calculated water temperature can be used at all times when the same mixer and the same weight of dough are used, for desired temperature of the dough in mixing process. Temperature deviations can influence the forming of volatile compounds in dough mixing time.

Table 1. Temperatures of added water and mixed dough

Sample code	Required dough temperature, °C	Added water temperature, °C	Mixed dough temperature, °C
6-20	20	14.00±0.26	21.43±0.25
6-25	25	22.10±0.26	25.07±0.40
6-32	32	43.17±0.23	32.10±0.36
8-20	20	12.60±0.26	22.00±0.44
8-25	25	21.47±0.06	25.50±0.20
8-32	32	42.00±0.00	31.57±0.21
10-20	20	12.77±0.06	21.80±0.26
10-25	25	20.77±0.21	26.23±0.49
10-32	32	42.17±0.15	32.30±0.26
15-20	20	12.77±0.06	21.80±0.26
15-25	25	20.77±0.21	26.23±0.49
15-32	32	42.17±0.15	32.30±0.26

Results are given as mean ± standard deviation (n = 3)

Cereal grains contain important flavour precursors, such as amino acids, fatty acids and phenolic compounds, which produce various flavours during processing – mixing, fermenting and baking (Hansen and Schieberle, 2005). All volatile compounds that were found in 6, 8, 10 and 15 minutes of dough mixing at various temperatures are shown in Table 2.

Table 2. Composition of volatile compounds in mixed flour blend dough

Compounds	Peak area ($\times 10^6$) depending on dough mixing time and temperature											
	6-20	8-20	10-20	15-20	6-25	8-25	10-25	15-25	6-32	8-32	10-32	15-32
4-penten-2-ol	2.90	6.12	7.17	4.18	6.26	9.60	11.10	19.82	17.64	21.81	27.36	29.95
Hexanal	0.29	0.35	0.53	0.54	0.52	0.59	0.66	0.72	0.71	0.75	0.91	0.85
2-methyl-1-propanol	*n.d.*	0.06	0.18	0.90	*n.d.*	0.18	0.29	0.66	0.28	0.54	0.86	1.46
1-penten-3-ol	*n.d.*	*n.d.*	0.10	0.13	*n.d.*	0.04	0.13	0.08	*n.d.*	0.07	0.25	0.31
D-limonene	0.12	0.17	0.18	0.21	0.25	0.26	0.31	0.31	0.33	0.34	0.32	0.34
3-methyl-1-butanol	0.24	0.56	1.12	5.44	0.74	1.50	1.70	4.07	2.79	4.16	5.87	10.94
1-hexanol	0.73	1.08	1.66	2.78	1.22	1.64	1.69	3.36	1.89	2.74	3.38	4.14
1-octen-3-ol	0.04	0.09	0.09	0.11	0.07	0.13	0.20	0.22	0.12	0.21	0.31	0.34
Heptanol	0.02	0.04	0.05	0.06	0.04	0.06	0.09	0.15	0.06	0.14	0.17	0.26
Acetic acid	0.13	0.15	0.22	0.33	0.25	0.31	0.36	0.48	0.42	0.95	1.17	1.34
2-methyl-propanoic acid	0.08	0.15	0.18	0.20	0.11	0.17	0.22	0.26	0.17	0.19	0.19	0.24
(E)-3-nonen-1-ol	*n.d.*	*n.d.*	0.16	0.06	*n.d.*	0.15	0.17	0.21	0.11	0.26	0.33	0.34
Carvone	0.35	0.52	0.55	0.61	0.66	0.70	0.80	0.81	0.78	1.13	1.17	1.40

n.d. – not detected
Results are given as mean (n = 3)

Analysing the collected data, in all dough samples there were indentified 8 alcohols, 2 carboxylic acids, 2 terpenes and 1 aldehyde. In all samples there were found 10 common volatile compounds (4-penten-2-ol, hexanal, D-limonene, 3-methyl-1-butanol, 1-hexanol, 1-octen-3-ol, heptanol, acetic acid, 2-methylpropanoic acid and carvone). Three of volatile compounds found in flour blend were D-limonene (value of volatile compound peak area – 28.17×10^6), 1-hexanol (77.84×10^6) and heptanol (5.84×10^6). And these volatile compounds were found in mixed dough, too. D-limonene, 1-hexanol and heptanol in mixed dough show smaller volatile compounds peak areas comparing to peak areas detected in flour blend samples.

The peak areas of detected 13 volatile compounds increased in dough mixing process, when the mixing time and dough temperature increased. The added raw materials and their amount can effect aroma formation in bakery products, too (Theile et al., 2002). The volatile

compound peak area of D-limonene increased slightly. This could be explained by the fact, that the D-limonene can oxidize easily in moist air to produce carveol, carvone, and limonene oxide (Karlberg et al., 1992). In the mixed dough there was identified a volatile compound – carvone, where the peak area increased in mixing process from 6 min (0.35×10^6) to 15 min (1.40×10^6), by 1.05×10^6. Alcohol 2-methyl-1-propanol was detected in the sample 6-32, but 1-penten-3-ol was detected only in the sample 8-25 (Table 2). The grain itself contains certain flavour – active volatile compounds, such as aldehydes, alcohols and ketones, of course they contain important flour precursors, too, such as amino acids, fatty acids and phenolic compounds, which produce various flavours during processing (Hansen and Schieberle, 2005). The highest value of peak area (29.95×10^6) among all detected volatile compounds had 4-penten-2-ol (alcohol) indentified after 15 min of dough mixing at temperature 32 °C.

Most of these compounds are well known to affect bread flavour and their origin is either associated with yeast, or they can be produced in dough mixing, fermentation and baking process. All of the detected volatile compounds can produce dough aroma: 3-methyl-1-butanol can be formed in yeast secondary metabolism, which gives malty flavour to dough, 2-methyl-1-propanol produces whiskey odour, 4-penten-2-ol – fruity, 1-hexanol – freshly mown grass, D-limonene and carvone – caraway odour (Schieberle, 1996; Kulp et al., 2003; Sabovics et al., 2011).

CONCLUSIONS

Solid-phase microextraction in combination with GC/MS can be used for detection of volatile compounds in mixed dough, changing mixing times and temperatures.

To make dough in mixing process with required temperature (20±2, 25±2 and 32±2 ºC) the water temperature should be around 14, 21 and 42 ºC, respectively. The calculated water temperature can be used at all times when the same mixer and the same weight of dough are used.

In all mixed dough samples totally 13 volatile compounds were detected, among them 10 volatile compounds were common for all samples: 4-penten-2-ol, hexanal, D-limonene, 3-methyl-1-butanol, 1-hexanol, 1-octen-3-ol, heptanol, acetic acid, carvone and 2-methylpropanoic acid.

Volatile compounds detected in mixed dough did not significantly differ at the same temperature, changing mixing times (6, 8, 10 and 15 min), but they significantly differ at the same mixing times, changing dough temperatures (20, 25 and 32 °C), except D-limonene, which did not significantly differ in both cases.

ACKNOWLEDGEMENTS

This research has been prepared within the framework of the ESF Project „Formation of the Research Group in Food Science", Contract Nr. 2009/0232/1DP/1.1.1.2.0/09/APIA/VIAA/122.

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DETERMINATION OF THE WATER APPARENT DIFFUSIVITY COEFFICIENTS DURING OSMOTIC DEHYDRATION OF CARROT IN SUGAR BEET MOLASSES

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ABSTRACT: The objective of this research was to determine influence of different concentration of sugar beet molasses and the duration of the process of osmotic dehydration of carrot on apparent diffusion coefficients of water (D_w). Carrot samples were cut into cubes and immersed in different concentrations of sugar beet molasses (40.0%, 60.0% and 80.0% dry matter) during maximim process time of 5 hours. After each sampling time (60, 180 and 300 min) the carrot samples were weighed and analyzed for dry matter content. Solution concentration and immersion time had a significant influence on the D_w ($p<0.05$) at 95% confidence limit. Proposed equation that describes dependence of the concentration of osmotic solution and the time of osmotic dehydration on the apparent diffusion coefficient, can be successfully used for modeling process of osmotic dehydration of carrots in molasses solutions.
Key words: *osmotic dehydration, water apparent diffusivity coefficient, carrot, sugar beet molasses*

INTRODUCTION

Either fresh or processed, carrot *(Daucus carota)* is one of the most often used vegetable in human nutrition, primarily due to its nutrient, vitamin and dietetic value. Drying of carrot is very common way of its preservation. Dried carrot is a component of different mixed spices, instant soups, and already prepared meals. Pulverized dried are used as a supplement to various bakery products and cakes (Filipčev et al., 2010).
Osmotic dehydration (OD) is a mild process, which starts to be interesting because of the low processing temperature and energy requirements. OD is governed by osmotic pressure difference between the food material (hypotonic medium) and concentrated osmotic solution (hypertonic medium). The diffusion of water is accompanied by the simultaneous counter diffusion of solute from the osmotic solution into the tissue. Since the membrane responsible for osmotic transport is not perfectly selective, other solutes present in the cells can also be leached into the osmotic solution (Rastogi and Raghavarao, 2004). The rate and dewatering degree of the material and changes in its chemical composition depend on the type of the osmotic solution used, the kind and the size of raw material, as well as the ratio of material to osmotic solution, temperature, duration of dehydration, agitation of liquid solution and type of apparatus. Rate of OD is the highest at the beginning of the process. It results from the largest difference of osmotic pressure between the osmotic solution and the cell sap of the material and small mass transfer resistance at this stage of the process (Moreira and Sereno, 2003).
For fruits and vegetables dehydration, the most commonly used osmotic agents are sucrose and sodium chloride and their combination. Glucose, fructose, maltodextrin and sorbitol also can be used for osmotic dehydration (Ispir and Togrul, 2009). Recent research has shown that use of sugar beet molasses as hypertonic solution improves OD processes (Koprivica et al., 2009; Lević et al., 2007).
The Response Surface Methodology (RSM) equations describe effects of the test variables on the observed responses, determine test variables interrelationships and represent the combined effect of all test variables in the observed responses, enabling the experimenter to make efficient exploration of the process (Mišljenović et al., in press).

The objective of this paper is to determine the influence of different concentration of sugar beet molasses and the duration of the process of OD of carrot on apparent diffusion coefficients of water, calculated by the method for determination of the apparent diffusion coefficients proposed by Azuara et al., 1992.

MATERIAL AND METHODS

Carrot samples were purchased at a local market in Novi Sad (Serbia) and stored at 4ºC until use. Initial moisture content, $X_{0,}$ was 89.94 %. Prior to the treatment, the carrots were thoroughly washed and cut into cubes, dimension 1x1x1 cm. The amount of 100 g of carrot cubes samples were prepared for each treatment. Different concentrations of sugar beet molasses (40.0%, 60.0% and 80.0% dry matter) were used as osmotic solution. Sugar beet molasses was obtained from the sugar factory Pećinci, Serbia. Initial dry matter content in sugar beet molasses was 82.23%. For the dilution of sugar beet molasses distilled water was used. In all experiments, a weight ratio of solution to carrot samples was 8:1, considered high enough to neglect concentration changes during the process. The carrot cubes were put in a glass jars with 800 g of molasses solution and the jars were placed in the heat chamber under atmospheric pressure at 55ºC and manually agitated at every 15 minutes.
Dehydration lasted 5 hours and samples were taken out of the osmotic solution at different times (60, 180, and 300 min). After removal, carrot samples were washed with water and gently blotted in order to remove the excessive water. The samples were weighed and analyzed for dry matter content. Dry matter content of the samples was determined by drying the material at 105ºC for 24h in a heat chamber (Instrumentaria Sutjeska, Serbia) and measuring the weight loss of the product, gravimetrically on a scale. The solid content of the osmotic solutions was determined by Abbe refractometer, Carl Zeis Jenna (AOAC, 2000).

Osmotic dehydration
In order to describe the mass transfer kinetics of the OD, experimental data from three key process variables are usually obtained: moisture content, change in weight and change in the soluble solids. Using these, dry matter content (DMC) and water loss (WL), were calculated for different solutions and processing times as described by Mišljenović et. al, (2010).

Apparent diffusion coefficient of water
Apparent diffusion coefficients of water (D) were determined from experimental results of WL (g of water/g initial sample weight (i.s.w.)) after period of time t. The time interval in which is used the proposed model was 300 minutes. The results of the WL from Table 1 were used for calculating time/water loss ratio. Values of D of WL during OD in three different concentrations of molasses solutions, and four different times of dehydration were calculated from the following equation:

$$D = \left(\frac{WL}{WL_{\infty}}\right)^2 \cdot \left(\frac{\pi \cdot r_e^2}{36 \cdot t}\right) \text{ [m}^2\text{/s]},$$

Equivalent radius of the samples r_e , is calculated for the cubical shapes where sample had dimensions of 1x1x1 cm, and the equivalent radius r_e was calculated to be 6.205 mm.

Response Surface Methodology
The RSM method was selected to estimate the main effect of the process variables on D, during the OD. The independent variables were osmotic time (X_2) and concentration of osmotic solution (X_1), and the dependent variables observed was the D. The experimental data used for the determination study were obtained using a custom design, with 9 runs. A model was fitted to the response surface generated by the experiment:

$$Y_1 = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{2} \beta_{ij} X_i X_j, \quad (1)$$

where: β_{kn} are constant regression coefficients.

Statistical analysis

Analysis of variance (ANOVA) and response surface regression method (RSM) were performed using StatSoft Statistica, for Windows, ver. 10 program. The model was obtained for each dependent variable (or response) where factors were rejected when their significance level was less than 90%. The same program was used for generation of graphs and contour plots.

RESULTS AND DISCUSSION

The essence of the OD process is to obtain high DMC in the treated samples in order to increase microbiological and enzymatic stability. In the Table 1. values of DMC, WL, equilibrium water loss (WL_∞) and D for OD of carrot in all osmotic solutions are shown.

Table 1. Values of Dry matter content, Water loss, Equilibrium water loss and apparent diffusion coefficient for osmotic dehydration of carrot

	Concentration of molasses solutions (% dry matter)	Time of OD (s)	DMC (%)	WL (g of water/g i.s.w.)	WL_∞ (g of water/g of sample)	D (10^{10} m^2/s)
	X_1	X_2	Initial=10.06			Y_1
1	40	3600	22.49	0.3103	0.6353	2.24[a]
2		10800	30.34	0.4779		1.76[b]
3		18000	36.44	0.5325		1.31[c]
4	60	3600	25.36	0.4370	0.8764	2.32[a]
5		10800	43.96	0.6696		1.82[b]
6		18000	51.37	0.7254		1.28[c]
7	80	3600	27.03	0.5312	0.9461	2.94[d]
8		10800	53.30	0.7672		2.04[e]
9		18000	64.66	0.8174		1.39[c]

[abc] different letters in the superscript in the same column indicate significant statistical difference between the values, at level of significance $p<0.05$

The increase in concentration and immersion time during the OD resulted in higher content of dry matter in the samples. The highest value of DMC in carrot samples, after 5 hours, (64.66 %) was achieved when 80% solid content sugar beet molasses was used as the osmotic solution. With this relative simple food preservation method, low energy required and nutritionally favorable, DMC were increased several times, which implicate that osmodehydrated carrot can be used as a raw material in the different food processing or could be suitable pre-treatment for other preservation technique (convective drying, freeze drying…) (Pavkov et al., 2008). It was observed that concentration and dehydration time have important effect on the carrot dehydration rate. Rapid loss of water in the beginning is apparently due to the large osmotic driving force between the dilute sap of the fresh carrot and the surrounding hypertonic solution. Higher concentrations of molasses increased the osmotic pressure gradient and, hence, higher WL. The highest WL (0.8173 g/g of i.s.w.) was observed in the sample which was dehydrated in molasses with 80% solid content for 5 hours.

The highest values of D were calculated for the WL in the samples of carrot dehydrated in the most concentrated osmotic solution (80%) during the shortest period of time (60 minutes). With the extension of the time of OD, values of D has started to decline, so the

value of D at the time of osmotic dehydration of 300 minutes was only 47.28% of the value of D during the dehydration time of 60 minutes.
The value of D for WL at the time of the dehydration for 60 minutes in the osmotic solution with concentration of 40% of dry matter was 76.19% of the value of D at the same duration of dehydration in the osmotic solution with concentration of 80% of dry matter. These results can be explained by higher driving force for water diffusion due to the higher difference of osmotic pressure between more concentrated osmotic solution and sample of carrot than the gradient of osmotic pressure between less concentrated osmotic solution and sample of carrot.
ANOVA test was conducted by StatSoft Statistica, ver. 10 to show the significant effects of the independent variables to D, and also is it significantly affected by the varying treatment combinations (Table 1). D was found significantly affected by both process variables, treatment time and concentration, at 95% confidence level. The main influential variable seems to be the treatment time ($F=295.48$, $p<0.01$), concentration term is also very influential ($F=26.79$, $p<0.05$). Quadratic term for treatment time was not significant ($F=0.46$, $p>0.05$), while quadratic term for concentration were found significant at $p<0.10$ level ($F=5.61$). The interrelation term was found statistically significant, at 95% confidence level ($F=13.75$, $p<0.05$). A high r^2 (0.991) is indicative that the variation was accounted and that the data fitted satisfactorily to the proposed model, and it was found very satisfactory and showed the good fitting of the model to experimental results. Tab. 2 shows the ANOVA calculation for the response second order polynomial (SOP) models, eq. (1) of apparent diffusion coefficient, and Table 3 shows regression coefficients for D.

Table 2. ANOVA table for system responces, showing the variables as a linear, quadratic and cross terms on each response variable

Term	Source	Sum of Squares			
		df	*DMC*	*WL*	*D*
Linear	Concentration	1	517.453*	0,105*	0,187*
	Time	1	1003.368*	0,106*	2,065*
Quadratic	Conc (quad)	1	2.816^{ns}	0,003**	0,039**
	Time (quad)	1	43.090**	0,013*	0,003 ^{ns}
Crossproduct	Time Conc	1	140.186*	0,001 ^{ns}	0,096*
Error	Error	17	15.357	0,001	0,021
r^2			99.108	99.600	99.131

*Significant at 95% confidence level,**Significant at 90% confidence level,
^{ns}Not significant

Table 3. Regression coefficients of the SOP model for D

β_0	$\beta_1 \cdot 10^2$	$\beta_{11} \cdot 10^4$	$\beta_2 \cdot 10^5$	$\beta_{22} \cdot 10^9$	$\beta_{12} \cdot 10^6$
2.78± 0.55*	-2.15± 1.81 ^{ns}	3.50± 1.48**	-3.36± 3.05^{ns}	7.72± 11.41 ^{ns}	-1.08± 0.29 **

*Significant at 95% confidence level,**Significant at 90% confidence level,
^{ns}Not significant

The contour plots developed from the approximating SOP model is shown on Fig. 1. The contour plot for D showed a saddle point configuration, and its value raised to the lower right corner of the plot, with the increase of concentration, and with lowering the treatment time.

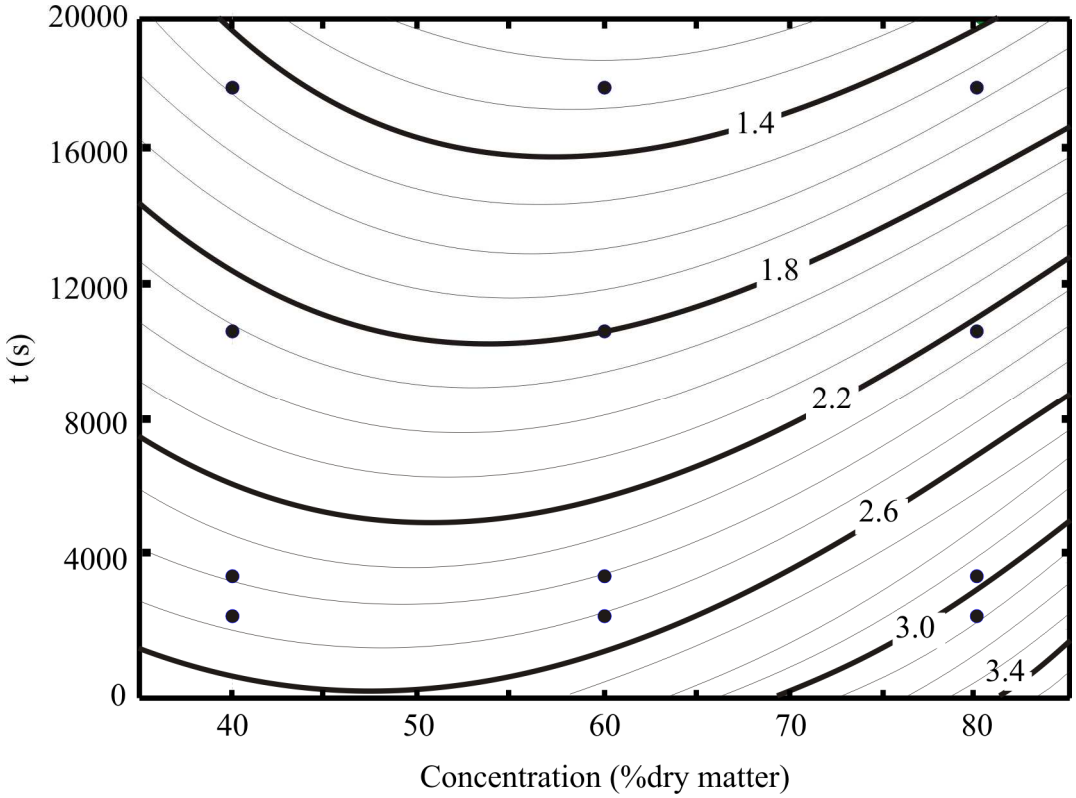

Figure 1. Contour plots for D e10 as a function of concentration of osmotic solution and treatment time time

CONCLUSION

The highest values of dry matter content (64.66 %) and water loss (0.8173 g/g of i.s.w.) were observed in the samples of carrot dehydrated in molasses with 80% solid content for 5 hours.
Values of the D of water from carrot in osmotic solutions of molasses ranged from $1.28 \cdot 10^{-10}$ m^2/s to $2.94 \cdot 10^{-10}$ m^2/s.
D was found significantly affected by all process variables, treatment time and concentration, at 95% confidence level.
Proposed equation that describes dependance of the concentration of osmotic solution and the time of osmotic dehydration on the apparent diffusion coefficient, due to the high coefficient of correlation can be successfully used for modeling process of osmotic dehydration of carrots in molasses solutions.
A new environmentally friendly value has been added to the molasses, wich is a by-product of sugar beet production, by extension of it's usage as a osmotic solution in a low energy required process of osmotic dehydration.

ACKNOWLEDGEMENT

These results are part of project supported by the Ministry of Education and Science of the Republic of Serbia, TR-31055, and 2011-2014.

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DEVELOPMENT OF INNOVATIVE TECHNOLOGIES FOR A HIGHER LEVEL OF BERRY FRUITS PROCESSING IN SERBIA

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ABSTRACT: In accordance with the national strategy for sustainable development, the objective of our investigation is to develop innovative technology for a higher level of raspberries processing and to obtain products for the food and confectionery industries, as well as the active ingredients for the functional foods and dietary supplements. The concept of food engineering is applied in the development and scale up of processes from laboratory to semi-industrial level. Key entities of the product quality for medical application are verified by antioxidant capacity, anti-microbial and anti-cancer effects. Incremental innovation is achieved in the process development and additive innovation in the design of technical solutions. A preliminary economic feasibility shows positive effects in relation to the raspberries costs for the higher level of processing, domestic market capacity and sales prices of competitive products. The current level of innovative technology and estimated economic indicators provide the basis for the development of industrial technology, competitive production and international technology transfer through a business model based on partnership relations with the use of development funds, loans and grants.
Key words: *berry fruits, higher level processing, food engineering, innovation, economic indicators*

INTRODUCTION

Serbia has a large quantity of natural and acquired raw material resources available in agriculture and in food industry. The natural raw material resources are exported or used for lower phase of preparation. The acquired resources are mostly used as waste material. The existing, available technological equipment capacities of chemical, food and pharmaceutical industries (Hranisavljević, 2007), would be used for manufacturing bulk products for food and confectionery industries and the medical substances for functional foods and dietary supplements.

The fruit processing chain is at a low level within the current development and a food product range. Serbia exports fruit as frozen products, processed at the lowest level (Hranisavljević, 2010). In the case of the berry fruits (raspberries, blackberries, etc…) the products of a higher processing level are mostly imported.

On a global scale, raspberries are very important in many different fields, from food and confectionery to pharmaceutical and cosmetics products. Raspberries are a significant source of anthocyanins and quercetin, the powerful antioxidants. These decelerate the effects of aging, prevent cancer and may reduce the risk of heart disease. Quercetin also reduces the release of histamines, which may minimize allergic reaction. In addition, raspberries are a significant source of ellagic acid, a phenolic compound known to have potential anti-carcinogenic features (www.bremnerfoods.com). Raspberry seed oil has very important application. Red raspberry seed oil is a superb antioxidant. It contains 83% essential fatty acids, especially high levels of Omega-3 and Omega-6 acids, and exceptionally high levels of alpha and gamma tocopherols, Vitamin E. Thus, the red raspberry oil is known especially for the prevention of gingivitis rashes, eczema and other skin lesion and sun protection. It is useful in skin creams, bath oils and tooth paste (www.man-gmbh.com).

Domestic production of raspberry's products is poorly represented and is carried out according to traditional methods and with small production capacities. During 2010, Serbia exported about 80% of frozen and fresh raspberry, while the remaining stock was used for the production of jams, concentrates, juices, etc... Over the years, the production of jams, marmalades, purees and pastes has been increasing. Serbia exported about 53 tones of them in 2010 (www.trademap.org).
In accordance with the National strategy for sustainable development, the aim of the case study presented in this paper is a part of our investigation (Hranisavljević, 2011) to develop innovative technologies for a higher level of raspberries processing and to obtain bulk products for the food and confectionery industries (juice concentrate, natural aroma, natural colors), as well as active ingredients for functional foods and dietary supplements (seeds, pectin).

MATERIAL AND METHODS

The process engineering concept (Kolomejcova, 2001; Mahoney, 2006) is applied in the innovative development for higher level of raspberries preparation.
Incremental innovation is achieved in the key process development and additive innovation in the design of technical solutions.
Key entities of the product quality were dry material content and pectin concentration. In the case of medical application, the products quality will be verified by antioxidant capacity, anti-microbial and anti-cancer effects analysis (Hranisavljević, 2011-2015).
A preliminary economic effect was defined in relation to the raspberries costs for the higher level of processing, domestic market capacity and sales prices of competitive products.
The business model based on partnership between the research Institutes and the University is applied.

RESULTS AND DISCUSSION

The technologies are developed for a higher level of the raspberry processing with the aim of obtaining products of various profiles (Table 1)

Table 1. Product profiles of raspberries at the higher processing level

Product name	Product Category	Use of product
aroma concentrate	final semi-product	natural aroma juice concentrate with aroma
juice concentrate	final semi-product	natural colors no aroma juice concentrate with aroma
juice concentrate with Aroma	final semi-product	natural colors with aroma syrup
protopectin	semi-product	syrup, jam, jelly
seeds powder	final	dietary supplement

The eco-friendly technologies are developed. Thus, seeds and aroma are obtained as products and not treated as waste. Processes and technical solutions enable utilization of all the active ingredients from the raspberry fruit and integrated outcome of a several final products of processing (Figure 1).

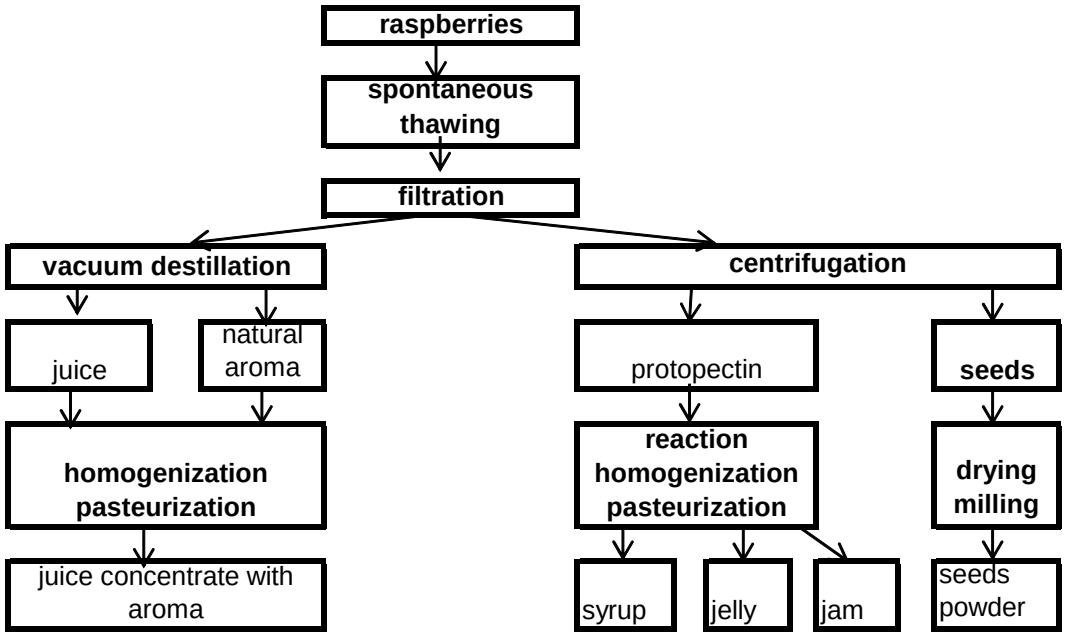

Figure 1. Flow diagram for the higher level of raspberries processing

Spontaneous thawing of raspberry as a critical process is performed at the temperature of t=20-30 ^{0} C with the absence of oxygen and light. During this step the tanning reactions (enzymatic and non enzymatic) are carried out at the temperature of t = 20-25^{0}C resulting in a greater concentration of coloring substances (flavonoides), increased antioxidant capacity and biological activities of the products obtained.

The protopectin is reactant in the hidrolysis to the pectin production The reaction was carrried out at the temperature t = 85^{0}C and pH 3,2-3,5. At the same time the homogenization and pasteurization are performed. The native ingredients in raspberries obtain a higher viscosity and degree of gelling depend on the pectin concentration.

In all technological stages the temperature is lower than t = 85^0 C, thus reducing the denaturation of biologically active substances in raspberries. Short retention time of material flows at the target temperatures creates conditions for the energy efficient technologies.

A technical solution for condensation the aroma is designed. The 2-stage vacuum destillation of aroma-water mixture evaporation is applied. The destillation was carried out at vaccum of 740 mmHg and temperature t = 20-25^{0}C. The technical solution being easier to use, more favorable in terms of energy efficiency and more acceptable in terms of investment, compared to the traditional process of rectification.

The concept of modular technology is applied. The modules are formed with standard processing equipment in accordance with the technology for obtaining a target final product. (Figure1). The concept allows for the efficient use of available production capacity in the domestic food and pharmaceutical industries, and the contents of modules can also be applied in processing other types of berries.

A preliminary economic feasibility shows positive effects in the application of the concept of integrated obtaining the products (Table 1, Figure 1). Based on the achieved material balance for the amount of final products and semi-products from 1kg of raspberries and the selling prices for the same amount of imported final products, the share of the raspberries purchase price in the aggregate selling price of the product is 1-3%.

CONCLUSIONS

Incremental innovation was achieved for the higher level of raspberries processing by using the integral concept at semi-industrial scale.
The modular technological concept was applied and additive innovation for existing technological equipment was achieved.
Non-waste and energy efficient technologies were developed, thus maintaining active ingredients of raspberries.
It is possible to develop a pilot-sample product at a higher level of the raspberry processing for investigating the domestic market with the aim of substituting the product import.
The final product for the food and confectionery industries (juice concentrate, natural aroma, natural colors), as well as the active substances for dietary supplements and functional food (seeds, pectine) were obtained.
Under the conditions of integrated output of the final products, the material balance achieved shows economically efficient participation of the raspberry price in the selling prices of imported products.
The achieved level of innovative technology and the preliminary economic indicators establish a basis for the industrial development of technologies and for competitive production.
Valorization of own knowledge could be possible via the development of technology export through the mechanisms of international technology transfers.
The innovation of a business model is possible through the strategic alliance and the cluster-based networking of the raspberry producers, small and medium-sized enterprises, as well as through the use of government and regional development funds, loans, and grants.

ACKNOWLEDGEMENTS

This paper is a results of research and development within the projects: TR-31093 "Technology for a higher level of berry fruits processing", and III-46001 ''Development and utilization of novel and traditional technologies in production of competitive food products with added value for national and global market-Creating wealth from wealth of Serbia", financed by the Ministry of Education and Science, Republic of Serbia

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EFFECT OF DRYING ON THE PHYSICAL PROPERTIES OF QUINCE

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ABSTRACT: This study aimed at evaluating the textural properties of quince in fresh and after drying at different conditions. In parallel, colour was also measured to evaluate how the visual aspect of the product changed with drying. The experiments were done in a tunnel drier, at different temperatures, from 30 to 60ºC, and different air velocities, varying in the range 0.7 to 1.2 m/s. The colour coordinates of the fresh quince were: L* = 78.02±0.86, a* = - 1.27±0.32 and b* = 31.75±1.68. These values indicate that the samples were bright, because L* was closer to 100 than to 0. Furthermore, it can be seen that quince was greenish, although very slightly and strongly yellowish. With drying, the samples became darker and redness was intensified, as a result of browning. In relation to the textural properties in fresh, the values were: hardness = 48.46±6.47 (N), springiness = 74.86±5.17 (%), cohesiveness = 0.75±0.06, resilience = 0.47±0.08 and chewiness = 27.23±4.06 (N). Furthermore, it was observed that drying, regardless of the conditions, induced an important hardening, increasing hardness and diminishing springiness.
Key words: *quince, drying, texture, colour, hardness*

INTRODUCTION

Quince is characterized by many flavour compounds derived from oxidative degradation of carotenoids, with the majority of them belonging to the class of thirteen carbon (C_{13}) norisoprenoids (Lutz-Roder et al., 2002). In addition to their potent antioxidant activity, it has an anti-inflammatory effect of a non-toxic, cost-effective natural agent (Essafi-Benkhadir et al., 2012). The chemical composition of its fruits (*Cydonia oblonga* Miller) range from 11.5 to 14.7 ºBrix, with fructose and glucose as predominant sugars, malic acid (0.78 %) as the main organic acid, followed by tartaric (0.22 %) and citric acids (0.009–0.014 %), besides having a low fat content. Furthermore, quince also is a source of dietary fibre, mostly pectic and cellulosic polysaccharides, and consequently it has been used in production of fibre rich powders (Pla et al., 2010; Thomas et al., 2000, Rodríguez-Guisado, 2009).
Besides its nutritional properties and health benefits, quince is used mostly for cooking preparations such as cookies, jellies, jams or marmalades since it is relatively hard, bitter and astringent (Silva et al., 2006). However, as the drying allows softening the pulp of the fruits, dried quince appears as an alternative to its fresh consumption. Different methods of drying are used to dry fruits and vegetables but all of them have a potential impact on the quality of the dehydrated products, namely on colour and texture properties. Therefore, the evaluation of both characteristics after drying is a pivotal aspect, since there is a strong impact on acceptability by consumers. Many works in the literature have studied the impact of different drying methods on the quality and properties of different fruits and vegetables such as red pepper (Doymaz and Pala, 2002), blueberries (Shi et al., 2008), potato (Leeratanarak et al., 2006), banana (Prachayawarakorn et al., 2008), apple (Mandala et al., 2005), bell pepper and pumpkin (Guiné and Barroca, 2011, 2012), mushrooms and onions (Guiné and Barroca, 2011a). Even though there have been a few works on drying of quince (Kaya et al., 2007; Koç et al., 2008) none of them has analysed their quality attributes.
The present study aims to compare the colour and texture properties of the quince in fresh and after drying in a tunnel drier, at different temperatures, from 30 to 60 ºC, and different air velocities, varying in the range 0.7 to 1.2 m/s.

MATERIAL AND METHODS

Drying

Quince was washed and cut into slices which were placed in a tunnel drier (Tray Drier UOP-8, Armfield). Drying experiments were performed under different conditions, namely varying air velocity and temperature. The values tested for air velocity were 0.7, 0.9 and 1.2 m/s and the temperatures essayed were 40, 50 and 60 ºC.

Colour evaluation

The colour of the fresh and dried samples was measured using a handheld tristimulus colorimeter (Chroma Meter - CR-400, Konica Minolta). A CIE standard illuminant D65 was used for calibration and the colour coordinates L*a*b* of the CIELab colour space were determined. From the Cartesian coordinates (L*a*b*) the total colour difference (□E) was calculated by equation (1):

$$\Delta E = \sqrt{(L^*_0 - L^*)^2 + (a^*_0 - a^*)^2 + (b^*_0 - b^*)^2} \quad (1)$$

having the fresh product as reference, with coordinates L^*_0, a^*_0, b^*_0.

The Cartesian colour coordinates were then used to calculate the cylindrical coordinates, chroma and hue, by the following equations:

$$\text{Chroma} = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

$$\text{Hue}\,(^o) = \text{atan}\left(\frac{b^*}{a^*}\right) \quad (3)$$

For the colour determinations twenty samples were analysed for each state (fresh and different dryings) and the mean values and standard deviations were calculated for each set.

Texture measurements

Texture profile analysis (TPA) to all the samples was performed using a Texture Analyser (model TA.XT.Plus from Stable Micro Systems). The texture profile analysis was carried out by two compression cycles between parallel plates performed using a flat 75 mm diameter plunger, with a 5 second period of time between cycles. A force load cell of 5 kg was used and the test speed was 0.5 mm/s. TPAs were performed in 20 samples for each state, and the textural properties: hardness, springiness, resilience, cohesiveness, and chewiness were calculated after the following equations (see Figure 1):

$$\text{Hardness (N)} = F_1 \quad (4)$$

$$\text{Springiness (\%)} = \square T_2/\square T_1 * 100 \quad (5)$$

$$\text{Resilience} = A_2/A_1 \quad (6)$$

$$\text{Cohesiveness} = A_3/(A_1+A_2) \quad (7)$$

$$\text{Chewiness (N)} = F_1 * \square T_2/\square T_1 * A_3/(A_1+A_2) \quad (8)$$

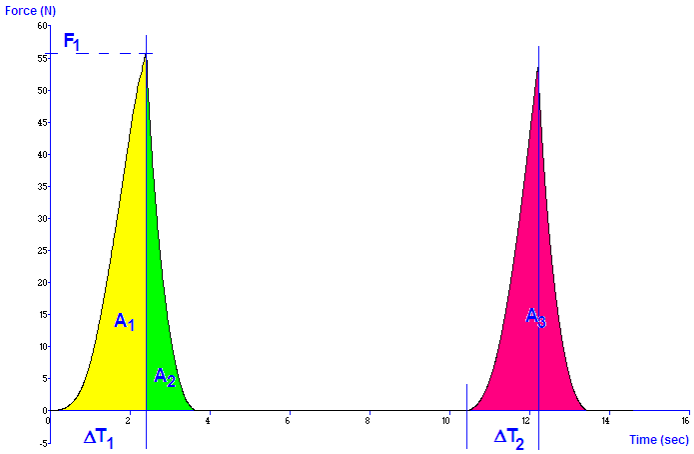

Figure 1. Example of a texture profile analysis.

RESULTS AND DISCUSSION

Figure 2 shows the variations through time of the experimental values measured for the Cartesian colour coordinates: L*, a* and b* for fresh and dried quince. The values of these coordinates indicate that the quince fresh pulp is clear (L* = 78.02±0.86), slightly green (a* = - 1.27±0.32), and very yellow (b* = 31.75±1.68). In general, drying produces a darker, redder and more yellow product when compared to the fresh product. Furthermore, it is possible to see that the increase in temperature from 40 ºC to 60 ºC had a small effect on the colour parameters since the brightness, L*, and the two opposing colour, a* and b*, ranged, respectively, from 74.41 to 71.80, 8.09 to 9.33 and 35.77 to 37.23.
For a temperature of 60 ºC, the increase in air velocity from 0.7 to 1.2 m/s also had a reduced effect on the colour parameters.

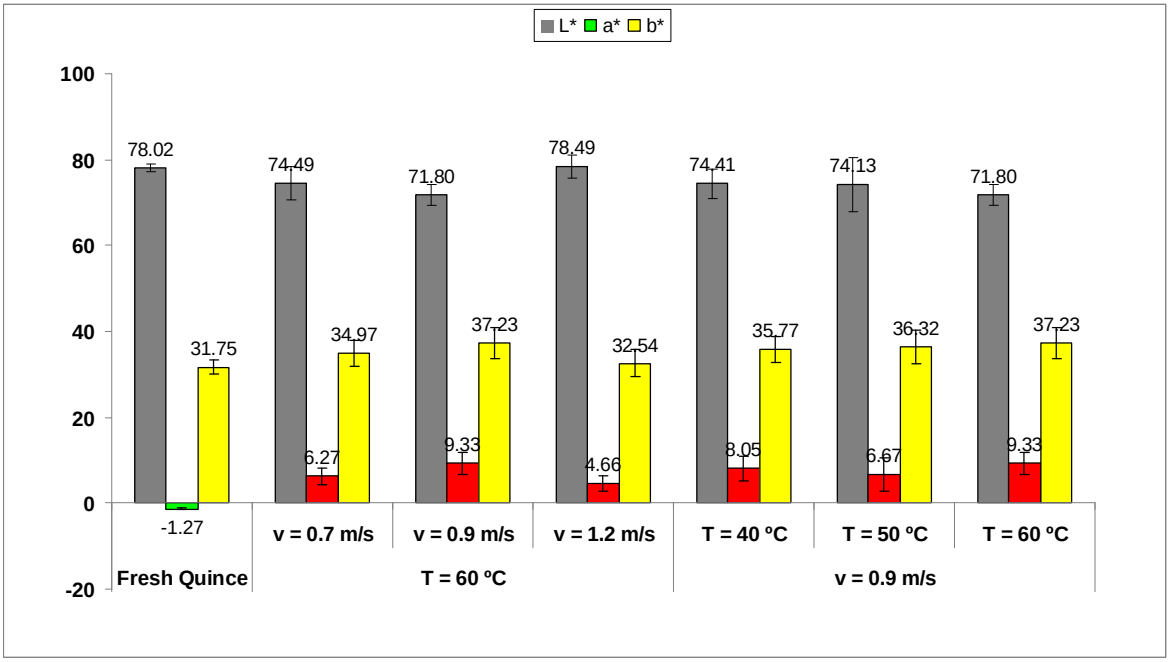

Figure 2. CIELab colour coordinates for fresh and dried quince.

Figure 3 reveals the total colour difference, which was calculated according to Equation (1), and quantifies the deviation of colour in relation to the reference colour. The range of temperatures and velocities studied induced a maximum colour difference of 13.45 in relation to the fresh quince, and this happened for the drying at 60 ºC and 0.9 m/s.

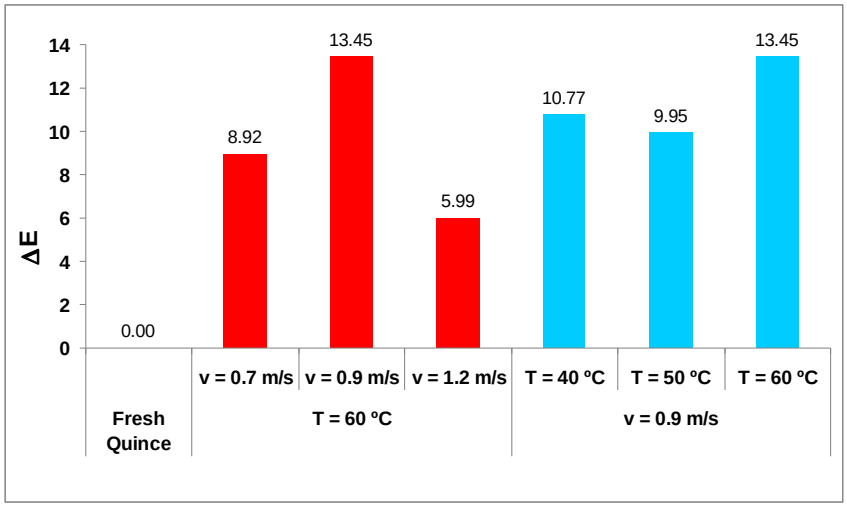

Figure 3. Total colour difference for dried quince samples.

Figure 4 presents the cylindrical coordinates, chroma and hue angle, calculated by Equations (2) and (3).The tunnel drying induces a small effect on intensification of colour (chroma), but moves the hue angle from the yellow colour to the red zone, although staying far from the fully red (0º), as compared with the fresh state. However, the operating factors temperature and air velocity had a slight effect on both cylindrical coordinates.

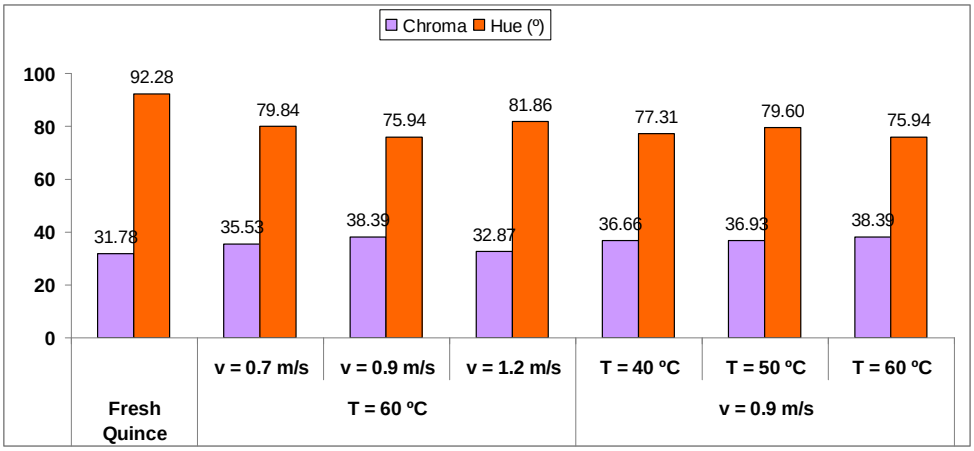

Figure 4. Cylindrical colour coordinates for fresh and dried quince.

Figure 5 highlights the results of hardness and chewiness obtained for the fresh and dried quince. It can be seen that the hardness of fresh quince is much lower than that of the dried state, independently of the operation conditions. This means that the drying intensifies the quince hardness and, consequently, the dried quince requires more energy than the fresh quince for the first bite. The hardness of fresh quince is 48.46 N against 87.26 N and 103.62 N, respectively, for 40 ºC with 0.9 m/s and 60 ºC with 1.2 m/s, which are the minimum and maximum values obtained after drying. However, the effect of drying on the energy required for chewing the quince until it is ready for swallowing, measured by the chewiness, is much less pronounced than in hardness.

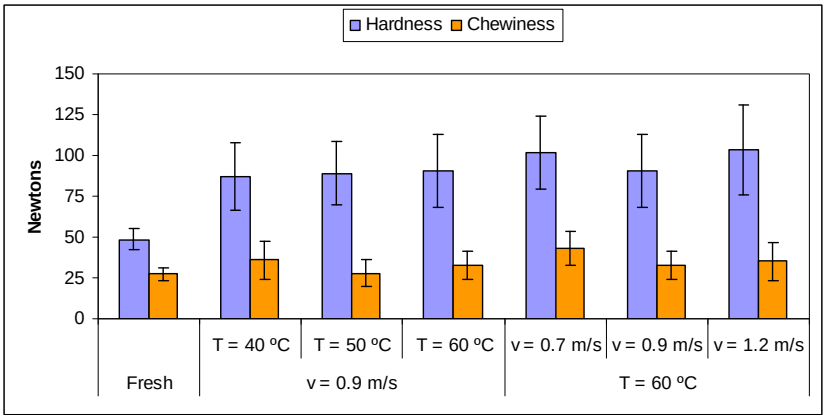

Figure 5. Hardness and chewiness of fresh and dried quince.

As seen in Figure 6, the drying has no visible effect on cohesiveness, which is related to the strength of the internal bonds of the sample neither on the capability of the sample to recover its size and shape after deformation (resilience).

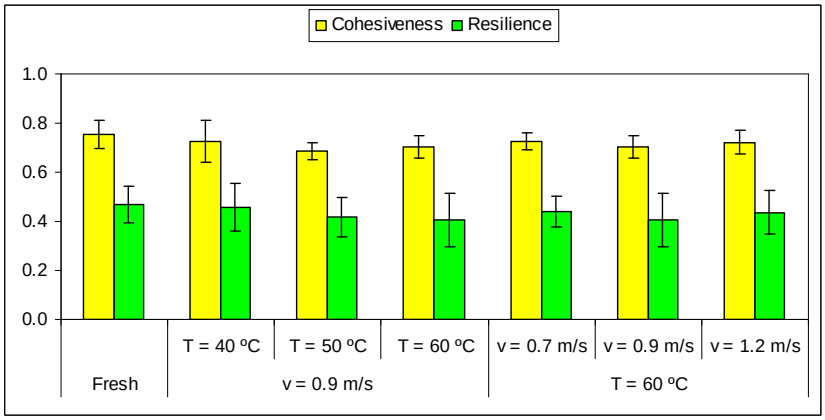

Figure 6. Cohesiveness and resilience of fresh and dried quince.

Figure 7 reveals that drying contributes to the reduction of the springiness of quince since the value of 74.86 % in fresh fruit is decreased to values near 50 % after drying.

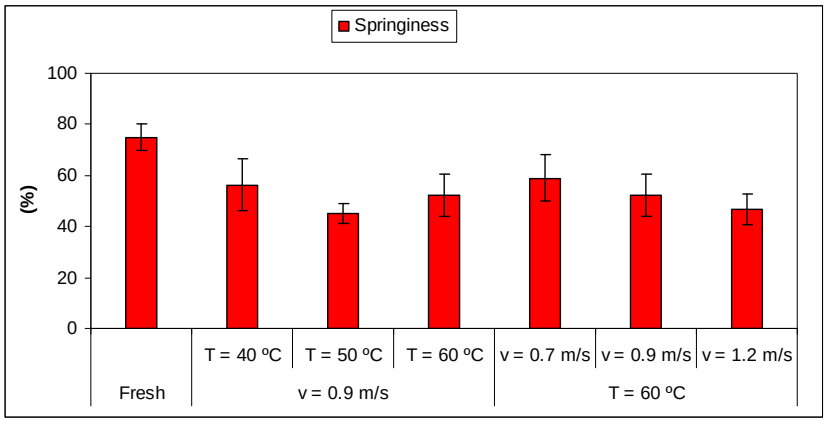

Figure 7. Springiness of fresh and dried quince.

CONCLUSIONS

Based on the results it was possible to conclude that tunnel drying produces a darker, redder and more yellow product, in relation to the fresh product, independently of the experimental conditions used. Besides that, the increase in temperature and air velocity had a slight effect on colour attributes (maximum colour difference, chroma and hue angle) in the range of temperatures and air velocities studied.

With respect to the textural attributes, it was observed that tunnel drying substantially influenced the hardness and springiness, regardless of the conditions, since induced an important increasing in hardness and a decreasing on springiness. The chewiness is another textural property that increases with drying, although not so strongly as hardness. The values of cohesiveness and resilience were not affected with drying, since the value in the fresh state is approximately the same as in the dried state, independently of the temperature and air velocity.

ACKNOWLEDGEMENTS

The authors thank the financial support to the research centres CI&DETS (Viseu) and CERNAS (Coimbra).

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EFFECT OF ELECTROMAGNETIC WAVES (MICROWAVE) ON MICROBIAL LOAD OF WHITE LIQUID STORED AT DIFFERENT TIME

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ABSTRACT: Pasteurized eggs exhibit better keeping quality and longer shelf life in comparison with un Pasteurized eggs. Also pasteurization guarantees the consumer's safety but thermal processing affects the egg proteins quality therefore in this study, microwave heating has been considered for white liquid egg pasteurization. White liquid was pasteurized by using electromagnetic method in two different frequencies (2950-4500 MHz) and both frequencies together for constant times of (3,5,6) second and two pulses (1,2), then with blank samples after different storage times (5, 15, 60, 180 and 360 days), the population of aerobic mesophilic bacteria, coliforms and molds & yeasts were measured. Results revealed that the effect of four variables include adding frequency, duration process, number of pulses and pasteurized product durability on reduction of total mesophilic aerobic count was statistically significant and the number of bacteria in the 360 th day in the pasteurized and blank specimens were $1/8\times10^4$ cfu/ml and $2/15\times10^6$ cfu/ml respectively. The number of coliforms in pasteurized samples were less than 10 pcs/mL and in the blank samples were $1/15\times10^4$ cfu/ml as the number of molds & yeasts in pasteurized samples were less than 30 pcs/mL while in the blank samples were $7/5\times10^3$ cfu / ml. According to Iranian National Standard, in electromagnetic method, liquid white shelf life has been defined 360 days which it is very ideal in comparison with thermal pasteurization (7d max) and blank sample (2d max).
Key words: *Electromagnetic waves (microwave), White liquid, Mesophilic bacteria, Coliforms, Molds and yeasts.*

INTRODUCTION

Eggs are one of the most commonly consumed food products e.g. Many of the dishes like Caesar salad, hollandaise or béarnaise sauce, mayonnaise, eggnog, ice creams, egg fortified beverages etc. (Mermelstein 2001). Eggs are potential hosts and carriers for pathogenic microbes like Salmonella enteritidis, because of their rich nutritive values therefore heat pasteurization is a best solution for controlling these pathogens. Egg is used as a vital ingredient in several foods, especially for its exceptional functional properties. These properties mainly depend on the egg proteins quality and because of proteins denaturation are severely affected by heating (Dev, 2007). Egg proteins are highly heat sensitive components which in turn high temperatures have an undesirable effect on their functional properties like whipability, foamability, foam stability etc. (Hank et al. 2001). Microwaves can be used to raise the temperature of in-shell eggs to the required pasteurization temperature in a few minutes. (Tajchakavit 1997). Microwaves are not ionising radiations but the dielectric properties of the microorganism (i.e. heat generated within the microorganism), itself enhance its destruction in a microwave environment. The microwave power distribution inside the shell eggs also seems to be well suited for uniform pasteurization (Datta et al, 2005).

There are very restricted researches on making microwave pasteurization viable for industrial use and limited literatures are available. Egg microwave pasteurization could make the process faster, continuous and the total operation could be completed in a few minutes. The shell egg appears ideally suited for pasteurization in a microwave environment (Fleischman 2004; Rehkopf 2005). This study examined the effect of cold pasteurization by electromagnetic waves (microwave) on liquid egg white and followed by comparing the population of total mesophilic aerobic bacteria, coliform and molds & yeasts after different storage times (5, 15, 60, 180 and 360 days).

MATERIAL AND METHODS

Sample preparation

Samples were selected from a fresh and healthy batch of egg and they were broken by egg breaker equipment, and were separated (yolk and white egg) then were filtered (to separate crust and small particles) and after that were homogenized. These were performed by selecting 190 samples of 100 g from 100kg liquid white randomly and they were filled in special poly ethylene bags automatically after filtering and were stored at 0 to 4ºC until experiments.

Instrumentals specifications

Electromagnetic pasteurizer which was able to work in variety of temperatures, times and frequencies, was used (model SCP150, Afra Sanat Kimia Mashhad co, Mashhad, Iran).

Methods

190 pcs of 100 g samples in accordance with those mentioned above were prepared and 60 samples randomly selected within the reservoir system was placed and the frequency of 2950 MHz as pasteurized in 10 samples in a pulse of 3 seconds -10 sample a pulse of 5 seconds -10 samples in a pulse of 6 seconds -10 sample two pulses 3 seconds (6 seconds) -10 of the two pulses 5 seconds (10 seconds) -10 of the two pulses 6 seconds (12 seconds) and Also randomly selected 60 samples exactly the same way and at a frequency of 4500 MHz was pasteurized. 60 samples with random acts of two frequencies were pasteurized and 10 samples as control samples (non-pasteurized) were selected. Furthermore the effect of storage times on samples microorganisms that were treated in the appropriate conditions (0-4℃) for a period of 5, 15, 60, 180, 360 days, were examined. At the designated time, 36 samples from 18 treatment of the (two repeats) and two control samples for microbial cultures transferred to the laboratory for cultivation with culture medium of plate count agar in 2 repeat, and cultivated in 30 ˚C for 72 hours according to national standard NO. 5272,13248, and Total Aerobic Mesophilic Count (TAMC) and with culture medium of violet red bile agar in 2 repeat, and cultivated in 37 ˚C for 24 hours according to national standard NO. 11166,13248, and Total coliforms and with culture medium of yeasts glucose agar in 2 repeat, and cultivated in 25 ˚C for 120 hours according to national standard NO. 10899, 13248 and molds & yeasts were determined.

Statistical analysis

Each treatment was analyzed as randomized complete block design with ten replications and the data were assessed by analysis of variance (ANOVA) and Duncan s multiple range test using MSTAT-C software program. Differences among treatments were tested with least significant difference (LSD) test ($P<0.05$). Besides, correlation analyses were preformed to clarify the relations among parameters considered in this study. Microsoft Excel 2007 was used to plot apparent.

RESULTS AND DISCUSSION

The effect of different frequency electromagnetic (microwave) and different times of the frequency on microorganisms

Microwave energy inactivates micro-organisms through thermal kill and also has enough potential to cause biological damages as well as alteration of the cell membrane and metabolic functions. This injurious effect on the vegetative cells and may induce additional lethality and impair the recovery during the subsequent time period of product storage (Huang, 1989). In this study, the two frequencies 2950, 4500 MHz and two (4500 +2950 MHz) with a pulse time of 3, 5, 6 and 2 pulses time 3.5, 6 seconds (6,10,12 seconds total times) on liquid egg white was investigated. Results showed that pasteurization with electromagnetic waves, the number of microorganisms which were present in the liquid egg white, were reduced to the convention. In the first test of the fifth day, the total mesophilic aerobic count in pasteurized and control samples were $2/05\times10^2$ and $7/2\times10^3$ cfu/ml, respectively and also coliforms and yeasts & molds were found in pasteurized samples (table 1). This indicates that in wave pasteurization, the number of total mesophilic aerobic bacteria, coliforms and yeasts & molds dropped to 1/55, 0/95 and 1/6 logarithmic cycles as high as the blank samples, respectively. The results confirmed the results of kozempel et al. (1999) which reported microwave energy has a significant effect on reduction of microbial population in egg yolk liquid (Kozempel *et al.,* 1999). The results also agreed with results of Dev et al. (2008) which has been considered microwave heating for in-shell egg pasteurization. First of all, the effects of temperature (0-62^{o}C) and frequency (200 MHz to 10 GHz) on the dielectric properties of egg components were investigated. Laboratory trials on microwave heating of in-shell eggs indicated that the heating rates of both albumen and yolk were similar. Therefore, microwave heating appeared perfectly suited for in-shell egg pasteurization (Dev *et al.,* 2008). According to results, that obtained from statistical analyzes, effect on adding frequency variants ($P<0.05$) and pasteurized product durability ($P<0.0001$) on reduction of total aerobic mesophilic count in white egg in α level of 0.05 (95% probability) and it was statistically significant, and interaction was observed between studied variants in cold pasteurization process. Storage time which was studied up to 360 days has a higher effect on total aerobic mesophilic count than various frequencies applied so the value of increasing TAMC was higher than reduction of it during white liquid pasteurization process by different electromagnetic waves. The results showed (Figure 1) the total of treatments, the frequency 4500 MHz with a total of two frequencies (4500 and 2950) was almost similar and the average number of bacteria in mesophilic aerobic was 3.6 cfu/ml log (4×10^3 cfu/ml), but the 2950 MHz frequency have poorer performance and aerobic mesophilic bacteria in the samples treated with the cfu/ml 9.3 log (8.3×10^3 cfu/ml), respectively. It showed that 2450 MHz frequency is better than 915 MHz frequency in pasteurization and sterilization, and higher frequencies will increase killing effects (Tang *et al.* 2002).

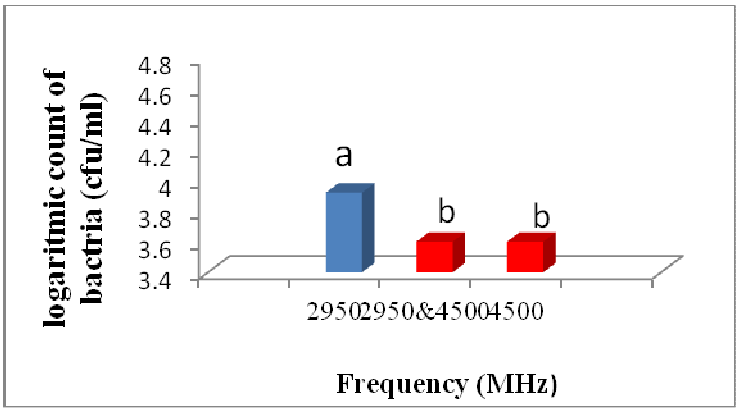

Figure 1.Effects of different frequencies on TAMC in liquid egg white cold pasteurization procedure

Effect of time process of the fatality of microorganisms and the short time pasteurization process and produce a higher quality product

Results also showed that the time process of the fatality rate is effective and often the higher frequency result in higher case fatality rate. The number of aerobic mesophilic bacteria at 3, 5, 6 respectively cfu/ml 3.63, 3.72, 3.83 is the logarithmic terms (Fig. 2). The higher pulse number dues to the higher fatality rate and lower total bacteria count (Fig. 3). Microwave sterilization can deliver products that taste good because microwaves are able to heat the product 3-5 times faster than conventional sterilization systems. The microwave-sterilized product is not temperature abused, so the food looks better, has better texture and better tastes than products processed by other available technologies (Harlfinger, 1992). There are significant reduction in process time which is important in results of other studies, also in this study confirmed total time of process is in most the time (time off and relax in two 6-second pulse) only 15 sec that it is very short comparing with 120 sec time of thermal pasteurization. And why do not adversely impact on the functional properties of egg white.

Tang and others (2010) Showed that it is possible to reduction packaged food pasteurization time from 1.1 to 1.4 in comparison with regular methods (Tang *et al.,* 2010). In comparison with regular methods for heating foodstuffs, pasteurization by microwave potentially could improve organoleptic properties, appearance and nutritional value. Ohlson (1987) reported that very short time length in microwave process causes to producing a product with very higher grade and quality comparing with products obtained from regular heating methods (Ohlson, 1987). Also David Reznik (1995) showed that there are many obstacles in egg pasteurization because of its unique properties. Because of these contortions and according to trading practical respects, yolk liquid thermal pasteurization is very difficult (Reznik, 1995). Results of this research showed that usage of both heat and waves could have stronger effect on lethal factor that it confirmed Kozmpel et al. which demonstrated that microwave energy may complete or strength thermal effects (Kozmpel *et al.,* 1999). Such that Sanvo co. (2010) used microwave method combine with 65°C heating and claimed that this methods very quickly and total time of operations is only a part of second and product heated to only coagulation and coacolation point and consequently the product was completely like fresh egg. This process decreases total amount of bacteria 10 times more than regular pasteurization methods. Studies showed that the method will protect egg functional properties 20% more than regular methods (Colavitti *et al.,* 2010). In different studies have severally referred to damages to egg yolk physical and functional properties in temperatures above 60 °C, for instance Wang et.al. (2009) showed that thermal denaturation was affected by dielectric properties and polar compounds in egg, and these changes (higher loss coefficient) occur in temperatures higher than 60°C which is effective on egg yolk

functional properties and results in losing some of this properties (Wang *et al.,*2009). In a similar study by Huang egg white-containing mixture was inoculated with Escherichia coli (ATCC #25922) at a concentration of 10 cfu per ml, and subjected to microwave heating to various product temperatures. The resultant samples were tested to determine bacterial survival and water soluble protein content. An Arrhenius plot of the inactivation rate vs. temperature (1/T) was then constructed. The rate of bacterial kill increased at a greater pace than that of the protein denaturation. Based upon the study results, it is believed that the advantage of having bacterial killed by increasing the processing temperature outweighs the drawback of losing some protein functionality (Huang, 1989).

Table 1 - Treated samples by electromagnetic waves of compared with blank samples in growth of microorganisms (cfu/ml) during storage of egg whit

Day/results	**Total count (cfu/ml)**		**Coliforms (cfu/ml)**		**Molds/yeats (cfu/ml)**	
Storage (day)	**Blank sampel**	**Treatment sample**	**Blank sampel**	**Treatment sample**	**Blank sampel**	**Treatment sample**
5	$7/2\times10^{3}$	$2/05\times10^{2}$	9	0	40	0
15	$2/6\times10^{3}$	$6/2\times10^{2}$	85	0	$1/2\times10^{2}$	1
60	$6/05\times10^{4}$	$1/1\times10^{3}$	$3/5\times10^{2}$	1	$6/0\times10^{2}$	2
180	$3/9\times10^{5}$	$1/8\times10^{3}$	$1/0\times10^{3}$	2	$1/9\times10^{3}$	3
360	$2/15\times10^{6}$	$1/8\times10^{3}$	$1/15\times10^{4}$	6	$7/5\times10^{3}$	22

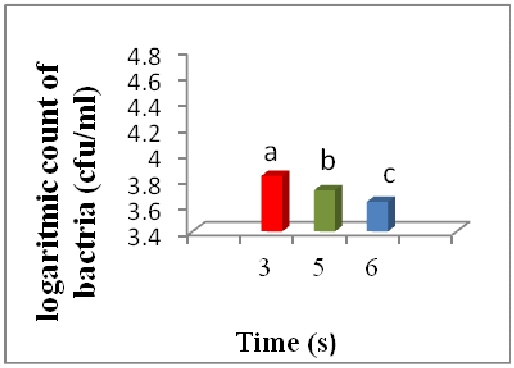

Figure 2**.**Effects of different time process on TAMC s6in liquid egg white cold pasteurization procedure

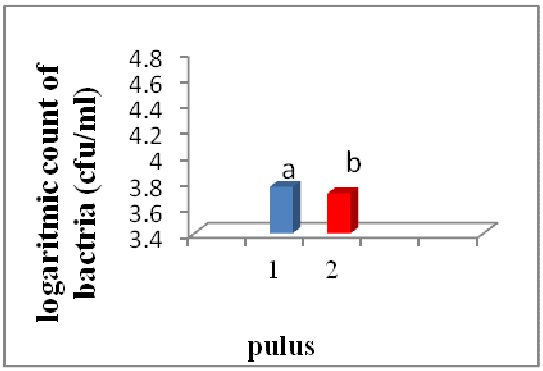

Figure 3**.**Effect of different pulses on TAMC

Determination pasteurized sample's storage time according to Iranian national standard limit

The number of aerobic mesophilic bacteria in pasteurized samples of the 2.31 log cfu / ml of the fifth day of 4.25 log cfu / ml in the 360 th day .This means that during the year, about 1/94 cycle logarithmic increase. Whereashe number of aerobic mesophilic bacteria in blank samples of the 3.86 log cfu / ml of the fifth day of 7.33

log cfu / ml in the 360 th day. This means that during the year, about 3.47 cycle logarithmic increase. Also the average number of coliforms count in the different treatments with pasteurized waves were less than 10 per ml and in blank samples wad the $1/15\times10^4$ cfu/ml in the 360 th day Also the average number of molds and yeasts count in the different treatments with pasteurized waves were less than 30 per ml and in blank samples wad the $7/5\times10^3$ cfu / ml in the 360 th day (table 1). Thus, according to Iran's national standard that limits the number of aerobic mesophilic bacteria in pasteurized products to conventional thermal ($3/0\times10^4$) and limits the number of coliforms (less than 10) per ml and limits the number of molds and yeats (less than 50) per ml and the products shelf life 7 days at(0-4 ℃) has been determined. Then concluded that pasteurized samples with wave after one year is still held. Therefore results of the study, confirmed Sanvo co. reports (2010) that invented commercial system for using waves in egg pasteurization, and announced that pasteurization by waves can increase eggs shelf life and protect its functional properties (Colavitti, 2010). Tang et al. (2002) in Washington university by similar studies on bean showed that the product was processed by microwave had better color and taste than those pasteurizing by regular method in cans and shelf life of sterile product by microwave will increase (Tang *et al.,* 2002).

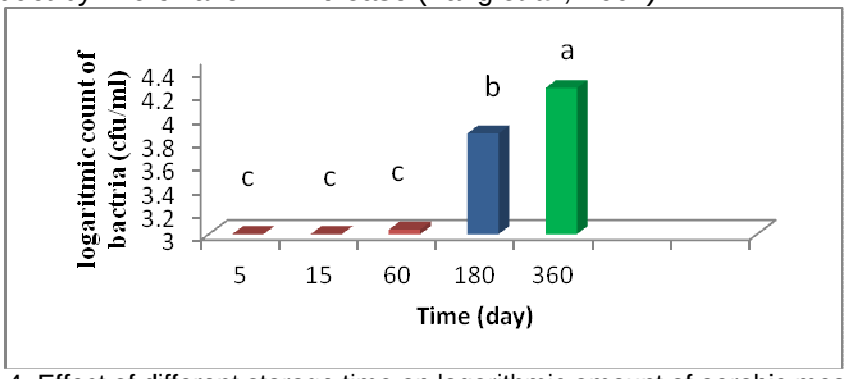

Figure 4. Effect of different storage time on logarithmic amount of aerobic mesophilic bacteria's in liquid egg white cold pasteurization procedure

CONCLUSIONS

Liquid egg white cold pasteurization significantly decreased total aerobic mesophilic count & other microorganism of samples. Therefore this method can be used in liquid egg yolk manufacturing industry. Because the egg whites are extremely sensitive to temperature and heating time on protein denaturation and loss of functional properties, it is very effective. So this method can be a safe and high quality product for use in food production. Shelf life of heat pasteurized liquid egg whites in the refrigerator temperature is a maximum of one month and In this new methods 12 times (about one year) increased then economic benefits of a reduction in production costs and maintenance and transportation for manufacturers and consumers have and less impact of price fluctuations are fresh eggs.

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EXTRUSION PROCESS TECHNOLOGY FOR FEED AND FOOD USED IN ANIMAL NUTRITION

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ABSTRACT: Deficit of food for population that grows every year, significantly makes the food production a very important problem the world and in Serbia. The solution to increase the feed and food production is in the usage of new technologies, technologies in biotechnology, actually in bioindustry. One of the main features of contemporary agriculture is production of feed of high nutritive value and microbiologically safe feed in sufficient quantities. The most important feedstuffs that are used in animal nutrition like cereals and other grain – soybean, rapeseed, corn, barley and sorghum, beside nutritive substances contain certain antinutritive substances, that represent limiting factor of their usage. Many antinutritive substances can be eliminated or significantly reduced with the usage of adequate heat treatments. The aim of this study was to collect, display and interpret the results, achievements and latest world science and technology trends trough application of contemporary technical and technological processes of feed processing. With the application of the latest heat treatment, such as extrusion, it comes to destruction of antinutritive substances and improvement of nutritive, hygienic, physical-chemical and other feed properties. The usage of processing technology such as extrusion is certainly one of the most important alternatives for feed and food production which by its high nutritive values can fulfill high demands of modern nutrition.
Key words: *feed, heat treatments, extrusion, animal nutrition.*

INTRODUCTION

Deficit of food for population, that grows every year, significantly makes the food production a very important problem the world and in Serbia. The solution to increase the feed and food production is in the usage of new technologies, technologies in biotechnology, actually in bioindustry. Numerous technological processes aimed at improving nutritional value of food and feed products intended for human and animal consumption and efficiently utilization of primary and processed agricultural and food byproducts have been developed worldwide (Filipović et al., 2010). Now days, huge numbers of various heat treatment processes are used all over the world for cereals and other grain processing, and among them is cooking, roasting, popping, steam flaking, toasting, conditioning, pelleting, micronization and extrusion (Marsman et al., 1998; Tsukamoto et al., 1995, Lević and Sredanović, 2010). According to the literature data, and practical experience, extrusion is the most commonly used process in Serbia for extruded products (Sakač et al., 2003; Sakač et al., 2006; Stanaćev et al., 2011; Puvača, 2011). Many raw materials in the basic form are not usable as feed for animals and nutritional value of certain raw materials is often lower than expected based on content of individual nutrients. Most often, the reason for

that are chemical and physical characteristics that reduce the biological value and digestibility of one or more ingredients. In order to enable the optimal use of nutrients, the technological processes in which the mixtures or raw materials for their production can be translated into usable, namely more usable forms of feed, are studied (Sredanović et al., 2005; Jovanović et al., 2006; Sredanović et al., 2007; Jovanović et al., 2009). Heat treatments have found a significant practical application among the most studied technological processes to improve the usable value of feed (Lević and Sredanović, 2010; Puvača, 2011). Properly applied heat treatment process has shown to reduce anti-nutritional factors to an acceptable level, enhance digestibility of some nutrients such as protein, oil, carbo hydrate, and improve sensory properties and microbiological quality of final product. Along with the antinutrient content reduction, thermolabile nutritive components must be preserved, therefore, process need to compromise these two demands (Jansen 1991; Vujković et al., 1993; Stanaćev et al., 2008; Filipović et al., 2010).
The aim of this study was to collect, display and interpret the results, achievements and latest world science and technology trends trough application of contemporary technical and technological processes of the most important feedstuffs such as oilseeds and cereals that are used in animal nutrition.

Extrusion proces

Extrusion is the process in which the feedstuff or mixture is pushed through the barrel by means of screws of different configurations and pressed through the die at the end of barrel. Extrusion is the technological treatment in which the material is exposed to high temperatures, up to 200˚C for 1-2 minutes or more precisely the material temperature increases progressively within last 15 to 20 seconds up to the optimum one to achieve the desired effects (Riaz, 2007). For that reasons, extrusion is a process classified as heat treatment with high temperatures and short period of its action. The feedstuffs for extrusion are also exposed to high pressure, which can range up to 25MPa (Bekrić et al., 1997; Jovanović et al., 2006; Kirchner, 2009). Extrusion is a complex and complicated technological process, but it is very flexible and provides the possibility for processing of a range of different raw materials such as oilseeds, cereals, legumes, raw materials with high moisture content, combinations of raw materials, by-products and wastes from food industry and complete animal feed mixtures (Smoje et al., 1996). Extrusion modifies the internal structure of feedstuffs. After treatment, the product is different, from nutritional point of view, compared to the raw material. Properly extruded material is much better, according to its nutritional and physical properties that the pelleted material. During extrusion process, feedstuffs passing through a whole range of changes, primarily in starch and protein components (Bekrić et al., 1997). However, beside positive effects of extrusion, irregular extrusion can result in negative effects (Lević et al., 1999).

Nutritive value of extruded feedstuffs

Soybean processing into high protein and high energy feed involves application of heat treatment process such as extrusion. Advantage of this process, the final result of which is full fat extruded soybean grits, soybean processed by dry or moist extrusion and heat treated soybean seeds, is that it offers possibility for the production of quality product with improved hygienic and physicochemical properties. Soybean seed has to be subjected to thermal treatment to inactivate or reduce inhibitory substances contained in soybean seed, such as trypsin and chimotrypsin inhibitor, chemaglutinin, phytate, saponin, antivitamin A, E, B12, so it could be used in animal and human nutrition (Bohm and Taufel, 1993; Verheul, 1997; Van der Poel, 1997). Quality of the feed made in dry extrusion process can be determined based on result obtained by Filipović et al., 2011, for chemical composition, amino acid composition and antinutritional factors of soybean seeds before and extruded full fat soy grits after treatment (Table 1).

Table 1. Quality indicators of soybean seeds and extruded full fat soy grits

Quality indicators	**Soybean seed**		**Extruded full fat soy grits**	
Chemical composition	% in sample	% in dry	% in sample	% in dry
Moisture	10.06	-	4.67	-
Crude protein	37.48	41.67	39.40	41.33
Crude fat	19.27	21.26	20.26	21.25
Crude fibre	4.39	4.88	4.08	4.28
Mineral matter	4.63	5.15	4.81	5.05
Amino acid composition (% in protein)				
Asparaganinic acid	12.00		10.35	
Treonine	4.59		3.63	
Serine	5.02		5.00	
Cystine	7.90		7.89	
Alanine	6.10		5.03	
Antinutritional factors				
Trypsin inhibitor (mg/g)	61.66		3.27	
Urease activity	10.95		0.26	
NSI (%)	65.82		25.64	

Filipović et al., 2001.

Rapeseed is an oilseed crop having a huge potential from an energy and protein standpoint, and containing components of high nutritive value. Beside proteins and oil, also contain antinutritional factors such as erucic acid and glucosinolates (Sakač et al., 2005; Sakač et al., 2007; Stanaćev et al., 2006; Stanaćev et al., 2008). Although rapeseed is a good source of quality protein, its use in rations is limited by its fibre, phytic acid, glucosinolates, polyphenols content. The use of rapeseed in compound feed is rather limited by its poor sensory properties because of high glucosinolate content, which is responsible for the pungent odour and biting taste particularly in non-ruminants nutrition (Stanaćev et al., 2011). When rapeseed is extruded alone its high oil content prevents adequate heating and produces an oily pulp which is susceptible to lipid oxidation. For this reason, rapeseed needs to be extruded in combination with other feedstuffs how the resultant product would have longer shelf life and be sustainable for storage (Stanaćev et al., 2006; Puvača, 2011). Reduction of total glucosinolates content, after dray extrusion process on 135˚C is shown in table 2.

Table 2. Glucosinolate content in rapeseed alone and in combinations with other feedstuffs before and after extrusion process

Sample	**Glucosinolate µmol/g DM (before extrusion)**	**Glucosinolate µmol/g DM (extruded)**	**Glucosinolate reduction (%)**
Rapeseed	65.91	46.43	29.56
Rapeseed + Corn 30:70	21.35	15.67	26.60
Rapeseed + Corn 50:50	32.83	24.62	25.01
Rapeseed + Barley 30:70	19.76	14.05	28.90
Rapeseed + Barley 50:50	33.57	25.35	24.49
Rapeseed + Triticale	23.34	16.56	29.05
Rapeseed + Triticale	36.73	27.86	24.15
Rapeseed + Wheat 30:70	19.80	15.25	22.98
Rapeseed + Wheat 50:50	41.70	32.26	22.64

Sakač et al., 2005

Corn is the most commonly used grain in the animal feed production in Serbia due to its high energy content (16.2 MJ/kg), relatively high oil content (3.9%) and low fibre content (2.2%) (Kovčin, 1993). In addition to its excellent digestibility, corn is considered to have better flavour than other grains. However, thermal treatments is used to enhance nutritional, hygienic, physico-chemical and other properties of grains, to improve nutritive value of some ingredients, upgrade sensory characteristics, ensure the microbiological wholesomeness of final product and inactivate thermo-labile anti-nutrients (Bekrić, 1999; Jansen 1991). Chemical characteristics of corn and corn extruded at 90 and 95˚C are given in table 3.

Table 3. Chemical composition of ground and extruded corn

Quality indicators	Corn	Corn extruded at	Corn extruded at
Crude protein	9.25	9.07	8.97
Crude fibre	3.45	2.47	2.80
Crude fat	4.80	2.08	2.66
NSI	13.11	6.06	5.88
Starch	70.90	67.06	64.98
Total sugar	1.00	3.99	4.12

Filipović et al., 2009.

Structure changes in protein, during the process of extrusion, leading to decrease in protein solubility (Filipović et al., 2003). Also starch is gelatinized and degraded and accessibility of enzymes to starch granules in the digestive tract is improved. Starch content in extruded corn is lower than in non-extruded corn, and as the result, total and reducing sugars content is increased, thus contributing to the change of sensory parameters.

CONCLUSION

At the end of the paper, it can be concluded that the extrusion of soy grain is one of the heat treatments, used to improve nutritional, hygienic and physico-chemical properties, and also to inactivate thermo labile antinutritients and upgrade sensory properties. When is extrusion of rapeseed in question it can be seen that thermal process led to reduction of glucosinolate content in extruded material in relation to untreated material. Extrusion of corn resulted in improved nutritional value, with increased sugar content due to changes in starch structure.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III046012 “Study of modern biotechnological methods in the production of animal feed in order to increase competitiveness, quality and safety of the feed”, financed by the Ministry of Science and Technological Development, Republic of Serbia.

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FIBERS AND CELL WALL CONTENT AND IN VITRO DIGESTIBILITY OF DIFFERENT MAIZE HYBRIDS

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ABSTRACT: Analysis of the fiber or cell wall present in forages is of major concern in ruminant nutrition because diets often contain large amounts of forage, and the fiber fraction affects both feed intake and animal performance. Research has revealed that plant genetics can affect the quality and digestibility of whole plant maize silage. All carbohydrates in plant feeds are grouped into: 1. Structural carbohydrates (carbohydrates of cell walls) including NDF (neutral detergent fibers-hemicellulose+cellulose+lignin), ADF (acid detergent fibers-cellulose+lignin), ADL (lignin), and 2. Non-structural carbohydrates-NFC (carbohydrates located inside the plant cell) made of starch, sugars and pectin. The aim of this present study was to observe quality parameters of ZP hybrids biomass with different genetic background for silage and to determine the relationship of these parameters, as well as, their effects on the digestibility of maize biomass dry matter. The contents of lignocelluloses fraction were determined by the modified Van Soest detergent method while *in vitro* digestibility of the whole plant was done by the Aufréré method. Obtained results showed that the NDF, ADF and ADL contents in the whole maize plant of the observed different ZP hybrids varied from 42.6% to 50.9%, 19.3% to 25.7%, and 1.6% to 2.5%, respectively. The difference in the digestibility of the dry matter of the whole plant between hybrids amounted to 10.4%. The differences in the contents of lignocelluloses fraction affected the differences in digestibility of dry matter.
Key words: *NFC, NDF, ADF, ADL, digestibility, maize hybrids*

INTRODUCTION

Maize is one of the most important naturally renewable carbohydrate raw materials. As a high-yielding carbohydrate plant, maize is very competitive in relation to other cereals (Radosavljevic et al., 2010). All carbohydrates in plant nutrients are grouped into: I structural carbohydrates (carbohydrates of cell walls), which include NDF (neutral detergent fibers - hemicellulose+cellulose+lignin), ADF (acid detergent fibers - cellulose+lignin), ADL (lignin) and II nonstructural carbohydrates - NFC (carbohydrates present in the plant cell content) that are made of starch, sugars and pectin (Jovanović et al., 1993). However, according Polakova et al. (2010) the NFC of substances comprises a diverse group of substances in terms of their composition and nutrient contents, excluding carbohydrates inherent to NDF. The NFC group of substances includes organic acids, monosaccharides, oligosaccharides, fructans, starch, pectin substances and □-glucans. There are numerous confirmations that the feeding ration with a high NFC content ferment and produce a great quantity of propionic acid in the rumen, which results in a higher insulin concentration in blood, i.e. in animal weight gain. Feeding rations rich in digestible fibers (hemicellulose and cellulose) produce acetic acid in the rumen and increase the growth hormone level in blood, which results in the increased milk production (Jovanović et al., 1993). Analysis of the fiber or cell wall present in forages is of major concern in ruminant nutrition because diets often contain large amounts of forage, and the fiber fraction affects both feed intake and animal performance. Research has revealed that plant genetics can affect the quality and digestibility of whole plant maize silage (Jung, 1997). Considering all stated, as well as, the fact that the digestibility does not depend on

the energy concentration, the objective of this present study was to observe quality parameters of the whole plant of ZP hybrids with different genetic background for silage and to determine the relationship of these parameters, as well as, their effects on the digestibility of maize biomass dry matter.

MATERIALS AND METHODS

The hybrids of the FAO maturity group 100-700 (ZP 161, ZP 388, ZP 434, ZP 555, ZP 677, ZP 704) were used in this study. The two-replicate trail was set up according to the randomized complete-block design in the experimental plot of the Maize Research Institute, Zemun Polje. The experimental plot size amounted to 21m^2, while sowing density was 60,000 plants per hectare. Plants of each replicate were harvested in the full waxy maturity stage from the area of 7m^2 (two inner rows), and yields of fresh biomass of the whole plants, plants without ears and ears were estimated. Five average plants per replicate were selected for further tests. Samples of the whole plants were cut and dried at 60^{o}C for 48h. In order to determine the content of dry matter the whole plant samples were ground in the 1-mm mesh mill. Then, the analysis of the absolute dry matter was done on the oven dry basis (105^{o}C for 12 h) in order to estimate the total dry matter. Moreover, the analysis of the content of forage fibers (NDF, ADF, ADL, cellulose, hemicellulose) was performed by the modified Van Soest detergent method (Van Soest, 1963). The method was modified by Mertens (1992). *In vitro* digestibility of the whole maize plant was done by the Aufréré method (Aufréré, 2006). This method is based on the hydrolysis of proteins of the whole plant in the pepsin acid solution (Merck 2000 FIP u/g Art 7190) at 40^{o}C for 24 h, and then on the hydrolysis of carbohydrates in the cellulase solution (cellulose Onozuka R10) in duration of 24 h. Methods applied in order to determine basic chemical content of the maize plant samples (contents of ash, protein and fat) were described in previously published paper (Radosacljević et al., 2000). The NFC content in the whole plant samples is calculated according to the formula: (%) NFC = 100%DM – ((%) crude protein + (%) NDF + (%) fat + (%) ash) (Polakova et al., 2010).

Statistical analysis of data

Data reported for quality parameters of ZP hybrids biomass was assessed by analyses of variance (ANOVA) and LSD multiple test was used for any significant differences at the $P<0.05$ level between the means. All the analyses were conducted using statistical software package STATISTICA 8.1. (StatSoft Inc. USA).

RESULTS AND DISCUSSION

Table 1 presents the NFC content of the whole plant and evaluation of significance of difference between the observed ZP maize hybrids. The results showed that the NFC content in the whole maize plant of the observed different ZP hybrids varied from 33.20% to 41.22%. The difference in the NFC content of the tested ZP hybrids was 8.02%. Differences in the contents of NFC were statistically significant between hybrids ZP 388, ZP 555, ZP 704, and between these and the ZP 161, ZP 434 and ZP 677. Differences in the contents of NFC were not statistically significant between the ZP 161, ZP 434 and ZP 677.

Table 1. Content of NFC of the whole plant of the ZP maize hybrids

Hybrids	NFC (%)
ZP 161	39.93^{d}
ZP 388	41.22^{a}
ZP 434	33.20^{d}
ZP 555	39.63^{b}
ZP 677	34.33^{d}
ZP 704	38.14^{c}
$LSD_{0.05}$	1.29

Means in the same column with different superscripts differ (p<0.05)

Data on the content of NDF, ADF, ADL, hemicellulose and cellulose are presented in Table 2. The results showed that the NDF, ADF, ADL, hemicellulose and cellulose contents in the whole maize plant of the observed different ZP hybrids varied from 42.57% to 50.84%, 19.32% to 25.74%, 1.63% to 2.51%, 23.26 to 26.07% and 17.69 to 23.62%, respectively. The differences in the content of NDF, ADF, ADL, hemicellulose and cellulose among observed ZP hybrids were 8.27%, 6.42%, 0.88%, 2.82% and 5.83%, respectively. Statistically significant differences in the NDF content of tested ZP hybrids were found, except between the ZP 434 and ZP 677, ZP 555 and ZP 704. Differences in ADF content were statistically significant except between hybrids ZP 161 and ZP 704. Statistically significant differences in the ADL case were found only between hybrids ZP 161 and ZP 555. The difference in ADL content was statistically significant only between hybrids ZP 161 and ZP 388. In other ZP hybrids these differences were not significant. Differences in hemicellulose content were not statistically significant between the ZP 161 and ZP 434 and ZP 677, and between ZP 388, ZP 555 and ZP 704. Statistically significant differences in cellulose content in the examined maize hybrids were found. The differences in cellulose content were not statistically significant between the ZP 161 and ZP 704.

Table 2. Content of forage fibres of the whole plant of the ZP maize hybrids

Hybrids	NDF (%)	ADF (%)	ADL (%)	Hemicellulose (%)	Cellulose (%)
ZP 161	49.08^{b}	23.73^{c}	2.51^{a}	25.35^{ab}	21.22^{c}
ZP 388	42.57^{d}	19.32^{e}	1.63^{b}	23.25^{c}	17.69^{e}
ZP 434	50.84^{a}	24.77^{b}	2.19^{ab}	26.07^{a}	22.58^{b}
ZP 555	45.35^{c}	22.05^{d}	1.63^{b}	23.26^{c}	20.42^{d}
ZP 677	50.83^{a}	25.74^{a}	2.12^{ab}	25.09^{b}	23.62^{a}
ZP 704	46.52^{c}	22.95^{c}	1.93^{ab}	23.57^{c}	21.02^{c}
$LSD_{0.05}$	1.22	0.84	0.64	0.77	0.59

Means in the same column with different superscripts differ (p<0.05)

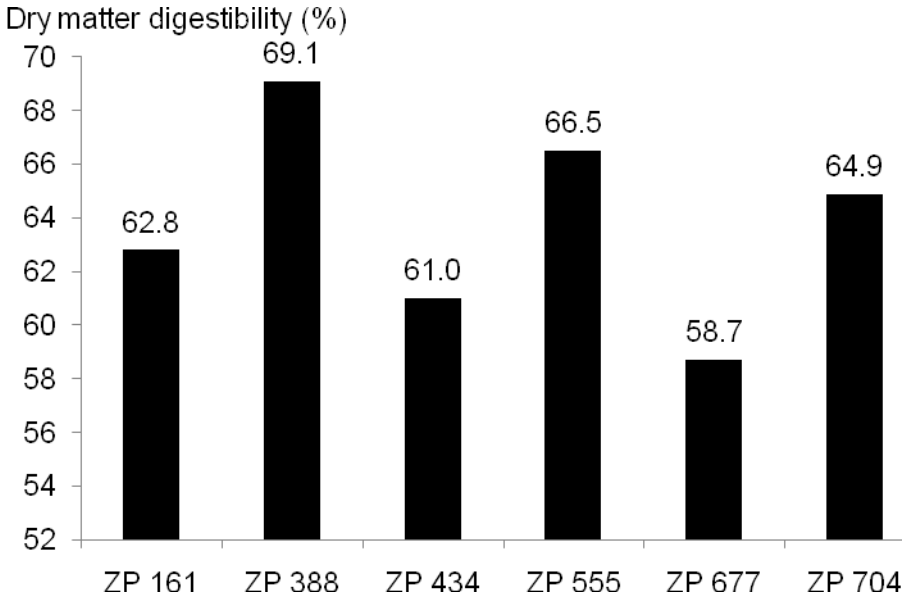
Considering the crucial effect of the NFC and forage fibers on the digestibility of the whole maize plant dry matter, the correlation dependence between the content of these components and the dry matter digestibility was observed (Table 3). A highly significant correlation between NFC content and dry matter digestibility of whole plant maize hybrids (r = 0.90), and a significant negative correlation between the content of NFC and the content of NDF, ADF, ADL, hemicellulose and cellulose were established (r = - 0.97, r = -0.90, r = -0.83, r = -0.96, r = -0.84). A very significant negative correlation was determined between the digestibility and NDF, ADF, hemicellulose and cellulose content (r=-0.96, r=-0.98, r=-0.79, r=-0.97), and significant negative correlation between the ADL content and the dry matter digestibility. Wermke (1986) ascertained a significant negative correlation (r=-0.67) between the digestibility and the ADF content, as well as, between the digestibility and lignin (r=-0.95). Burritt et al. (1985) studied three grass species and found high correlation dependence between the dry matter digestibility and the content of forage fibers. The coefficient of correlation between the digestibility and NDF, ADF, ADL and cellulose amounted to r=-0.84, r=-

0.93, r=-0.91 and r=-0.86, respectively (Burritt et al., 1985). Very significant positive correlation was determined between the NDF content and content of ADF, ADL, hemicelluloses and cellulose amounted to (r=0.97, r=0.74, r=0.90, r=0.94). Significant positive correlation was determined between content of ADF and content of ADL and hemicellulose amounted to (r=0.66, r=0.77) and very significant positive correlation with cellulose content (r=0.99). Positive significant correlation was determined also between content of ADL and hemicellulose (r=0.76) and also between hemicellulose and cellulose content (r=0.72). Wermke (1986) established that there were maize hybrids with a low digestibility and a high content of stover cell wall constituents, as well as, there were hybrids with a high digestibility and a low content of cell wall constituents. These hybrids were defined as a dislocation type of hybrids. Hybrids with a high digestibility and a low NDF content of stover and a high NDF content of stover and a low ear digestibility are classified into types of hybrids with prolonged stover assimilation.

Table 3. Correlation dependence between digestibility, NFC and forage fibres of the whole plant of the ZP maize hybrids

	NFC	NDF	ADF	ADL	Hemicellulose	Cellulose
Digestibility	0.90**	-0.96**	-0.98**	-0.64*	-0.79**	-0.97**
NFC		-0.97**	-0.90**	-0.83**	-0.96**	-0.84**
NDF			0.97**	0.74**	0.90**	0.94**
ADF				0.66*	0.77*	0.99**
ADL					0.76**	0.55
Hemicellulose						0.72**
Cellulose						

The Figure 1 presents results of the digestibility of the whole ZP maize hybrid plant dry matter. The digestibility of the whole ZP maize hybrid plant dry matter ranged from 58.7% to 69.1%. The difference in the digestibility of the whole maize plant dry matter amounted to 10.4%. According to results obtained by Deinum et al. (1981) and Andreu et al. (1974) this difference varied from 2% to 3%, while Terzić (2006) and Terzić (2010) established the highest difference among observed hybrids of 11.52% and 8.56%, respectively. Differences in the digestibility of whole plant corn were statistically significant between the ZP hybrids.



$LSD_{0.05} = 1.07$; $LSD_{0.01} = 1.68$

Figure 1. Dry matter digestibility of the whole plant of the ZP maize hybrids

CONCLUSION

Obtained results showed that the NFC content in the whole maize plant of the observed different ZP hybrids varied from 33.20% to 41.22%. The difference in the NFC content of the tested ZP hybrids was 8.02%. The results also showed that the NDF, ADF, ADL, hemicellulose and cellulose contents in the whole maize plant of the observed different ZP hybrids varied from 42.57% to 50.84%, 19.32% to 25.74%, 1.63% to 2.51%, 23.26 to 26.07% and 17.69 to 23.62%, respectively. The digestibility of the whole ZP maize hybrid plant dry matter ranged from 58.7% to 69.1%. The difference in the digestibility of the dry matter of the whole plant between hybrids amounted to 10.4%. The differences in the contents of NFC and forage fibers affected the differences in digestibility of dry matter.

ACKNOWLEDGEMENTS

This study was financially supported by the Ministry of Education and Science of Republic of Serbia, Project TR31068.

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IMPROVING THE TECHNOLOGY OF PRODUCING FOOD GRADE DYE FROM RED BEET JUICE

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ABSTRACT: Red beet juice is very useful food product, because it contains significant amount of sugars, mineral substances and vitamins. It is also valuable because it is used for producing food grade dye. Nowadays in order to give to food products a color, close to natural coloring of fruits and vegetables, expensive synthetic dyes are used, which might have cancer-inducing effect when being accumulated by human organism. Therefore improving the technology for producing food grade dye from red beet juice is remarkably important task. Currently for the purpose of obtaining dye from red beet juice, a part of pectin substances, which make the process of juice concentration more difficult, is removed with the help of expensive enzymatic agents. The authors have suggested using natural carbon-bearing adsorbent shungite to purify red beet juice from pectin substances.
Key words: *food grade dye, red beet juice, natural adsorbent, shungite*

INTRODUCTION

Red beet juice is very useful food product, because it contains significant amount of sugars, mineral substances and vitamins. It is also valuable because it's used for producing food grade dye.

Nowadays in order to give to food products a color, close to natural coloring of fruits and vegetables, expensive synthetic dyes are used, which might have cancer-inducing effect when being accumulated by human organism. Therefore improving the technology for producing food grade dye from red beet juice is remarkably important task.

Currently for the purpose of obtaining dye from red beet juice, a part of pectin substances, which make the process of juice concentration more difficult, is removed with the help of expensive enzymatic agents (Tymofeeva V.N. et al., 2002).

The authors have suggested using natural carbon-bearing adsorbent shungite to purify red beet juice from pectin substances (Sheiko and Melnyk, 2009; Sheiko and Melnyk, 2010).

Shungite is a mineral consisting of amorphous carbon and fractured graphite. Its chemical composition is not constant: shungite contains 60-70% of carbon and 30-40% of other elements.

Shungite is the only known mineral to have fullerenes (recently discovered new globular form of carbon existence). Fullerenes' structure is peculiar because carbon atoms in molecules are situated at the tops of regular pentagons and hexagons, which cover sphere's surface and present themselves as closed polygons composed of paired quantity of coordinated carbon atoms.

Fullerenes differ from particles with metallic properties due to the location of electron cloud and ability to change the form of carbon structure.

Sizing of electro-magnetic waves is determined by vibration of electrons which are divided into $\pi - \sigma$ and π- states. During adsorption on electrically neutral surface the localization of fullerenes' π-states takes place, and a particle loses its metallic

properties, and because of that connected electron pair appears in the activated form. Thus mineral shows bipolar properties.
Shungite's important characteristic is the presence of fullerene carbon nanotubes with the diameter of their cylindrical pores constituting 1-6 nanometers and the width - up to several micrometers. The cylindrical surface of tubes is formed by active carbon circles and also has empty pores.
The basis of shungite's structure is a globule composed of graphitic networks, formed into packages. Each package has 6 graphitic flat networks with the quantity of carbon atoms attaining to 300-600 and one curved network, having 400 carbon atoms.

MATERIAL AND METHODS

Shungite to be used for research was prior washed out with cold water and then termoactivated at 100℃ during 90 minutes. Cooled a dsorbent in concentrations of % mass.: 2.44; 3.23; 4.76; 9.09 was put into fresh red beet juice at temperature of 20, 40, 50, 60ºC, mixed during 10, 20, 30, 60 min., filtrated. The content of pectin substances in filtrate was measured in accordance with **calcium** pectate **method under formula:**

$$. \quad ПР = \frac{(g - g_0) \cdot 100 \cdot 0.9235}{V \cdot d}$$

whereas ПР – content of pectin substances in juice, mg/g;
g – weight of weighting cup with precipitate before exsiccation, g;
g0 – weight of empty weighting cup, g;
V – juice volume, cm3;
d – juice density, g/cm3;
0,9235 – coefficient for conversion of calcium pectate into pectic acid.

The obtained results were compared by effect of purification:

$$E = \frac{100 \cdot (K_1 - K_2)}{K_1}$$

whereas К1 and К2 – quantity of target component in juice which was not processed by adsorbent and juice which was processed by adsorbent.
The obtained results are shown in table below.

Table 1. Effect of purification (E,%) from pectin substances in red beet juice by shungite at different adsorbent concentrations, temperature of mixtures, duration of interactions between adsorbent and juice, initial content of pectin substances is 1.9 mg/g

Effect of purification	Adsorbent concentration in juice, % mass															
	2.44				3.23				4.76				9.09			
	Temperature, ºC															
	20	40	50	60	20	40	50	60	20	40	50	60	20	40	50	60
	Duration of juice processing, 10 min															
	Content of pectin substances, mg/g															
Processed juice	1.8	1.8	1.7	1.7	1.8	1.7	1.7	1.6	1.7	1.6	1.5	1.5	1.6	1.6	1.5	1.4
E, %	5.3	5.3	10.5	10.5	5.3	10.5	10.5	15.8	10.5	15.8	21.0	21.0	15.8	15.8	21.0	26.3
	Duration of juice processing, 20 min															
Processed juice	1.7	1.7	1.6	1.6	1.5	1.5	1.4	1.3	1.5	1.4	1.3	1.2	1.4	1.3	1.2	1.2
E, %	10.5	10.5	15.8	15.8	21.1	21.1	26.3	31.6	21.1	26.3	31.6	36.8	26.3	31.6	36.8	36.8
	Duration of juice processing, 30 min															
Processed juice	1.6	1.6	1.5	1.5	1.4	1.4	1.3	1.2	1.5	1.3	1.2	1.1	1.4	1.3	1.1	1.1
E, %	15.8	15.8	21.1	21.1	26.3	26.3	31.6	36.8	21.1	31.6	36.8	42.1	26.3	31.6	42.1	42.1
	Duration of juice processing, 60 min															

Processed juice	1.6	1.6	1.5	1.5	1.4	1.3	1.2	1.2	1.5	1.3	1.2	1.1	1.3	1.2	1.1	1.1
E, %	21.1	21.1	26.3	26.3	26.3	31.6	36.8	42.1	21.1	31.6	36.8	42.1	31.6	36.8	42.1	42.1

RESULTS AND DISCUSSIONS

The obtained data shows that adsorption of pectin substances from red beet juice by shungite takes place already at temperature of 20ºC. If temperature rises the process is somewhat accelerated. The rise of temperature of processing juice over 60ºC is unreasonable because coloring components are destroyed and that causes the changes in juice color.

Comparing the obtained results and their practical efficiency the authors determined optimal parameters, concluding that the optimal parameters for processing juice by shungite is adsorbent concentration of 4.76% mass, temperature of processing 50, 60º C, duration - 30 min. Under such conditions 36.8%-42.1% of pectin substances are removed.

The mechanism of adsorbing pectin substances from red beet juice by shungite is explained not only due to the fact that impurities infiltrate the mineral's pores, but also due to ion-exchange adsorption in places where reactive centers of fullerenes are formed and hydrogen bonds with pectin molecule are created.

Shungite's selectivity is explained not only because of the existence of micro-, mezzo- and macropores, but also because nanotubes participate in adsorption processes and there are pores in between them, created when packages are formed, and also because of free non-compensated charges which appear on adsorbent's surface.

The next phase of research was determining the content of coloring substances of red beet juice, processed by shungite at temperature of 50ºC. Preparation of shungite was performed in the same way as for adsorption of pectin substances. The content of coloring substances was determined according to standard methodology – reaction of Neubauer-Levental. Estimation of mass content of coloring substances was performed under formula:

$$X = \frac{(M_1 - M_2) \times K \times 0{,}004157 \times O_1 \times 100}{H \times O_2}$$

whereas X – mass content of coloring substances,%;

M1 – quantity of 0.1 normality of solution of potassium permanganate, used for the first titration, cm3;

M2 - quantity of 0.1 normality of solution of potassium permanganate, used for the second titration, cm3;

O1 – volume of the primary extraction, cm3;

H – quantity of juice used for experiment, cm3;

O2 – volume of the secondary extraction, used for titration, cm3;

0.004157 – coefficient which takes into account correlation between potassium permanganate and juice coloring substances.

The research was performed and its results are showed in figure 1.

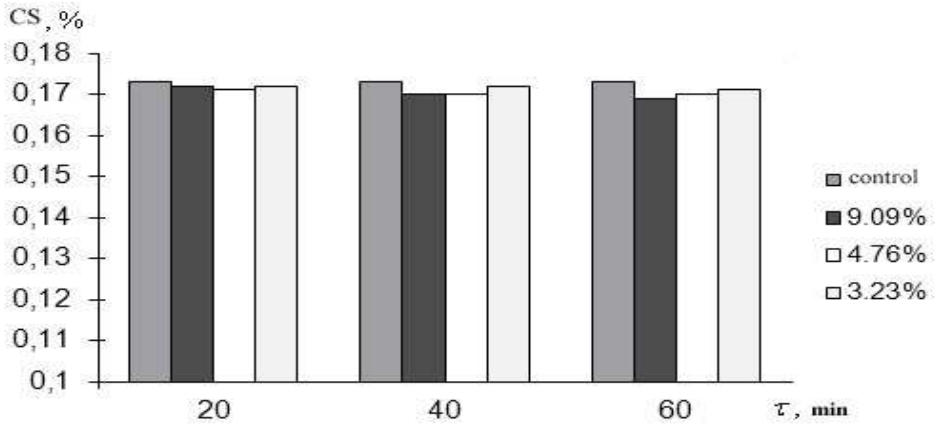

Figure 1. Quantity of coloring substances (CS) in juice processed with shungite depending on the duration of its processing at temperature of 50ºC.

Analysis of data presented in fig.1 gives grounds to state that content of coloring substances in red beet juice, after it was processed by shungite, practically does not change. This can be explained by the fact that the basis of coloring substances in red beet juice is anthocyanins (Saburov and Antonov, 1951; Tanchev S.S., 1980). By their structure they are chains of glicosides, composed of heterocyclic compounds. By their chemical nature they are surface active substances. On interphase border anthocyanins' molecules are situated in such a way that hydrophilic group remains in liquid state. Hydrophobic effect takes place and thus coloring substances are not adsorbed by shungite.

The authors improved apparatuses and technological scheme for producing food grade dye from red beet juice by installing two adsorbing devices with shungite which work in regime of sorption-desorption.

After part of pectin substances are removed in adsorbing device, red beet juice is placed in vacuum evaporator where it is concentrated around 6 hours. Concentration of juice by its evaporation takes place with discharge of 0.055 – 0.060 MPa and temperature of 55-60ºC, concentrated red beet juice, already as food grade dye, is packed in the sealed container, made of dark glass. The level of pH in the obtained food grade dye does not exceed 4.5.

The used adsorbent is sent for regeneration, utilization.

CONCLUSIONS

It was established that shungite effectively adsorbs pectin substances from red beet juice and does not adsorb coloring substances. The technology of producing food grade dye was improved by additional purification of red beet juice from pectin substances by shungite. The obtained optimal technological parameters for purifying juice with shungite are as follows: adsorbent's concentration constituting 4.76% mass., temperature is 50-60ºC, duration of processing is 30 min. Apparatuses and technological scheme of producing food grade dye is supplemented with two adsorbing devices with shungite, which work in regime of sorption-desorption. The used adsorbent is recommended to be regenerated, utilized.

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INFLUENCE OF HEAT TREATMENT IN WATER ON CHANGES OF TOMATO PROPERTIES DURING POSTHARVEST STORAGE AT LOW TEMPERATURE

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ABSTRACT: Storage of tomato after harvest at low temperature in order to prevent decay and prolong its postharvest shelf life induces chilling injuries and makes fruit susceptible to the colonization of microorganisms that can cause fruit deterioration. One of postharvest treatments used for reducing of chilling injuries is the heat treatment in water or in the air flow. In order to investigate influence of heat treatment in water on properties of tomato stored at low temperature (4℃) during three weeks, commercial tomato was harvested at mature green and red-ripe stages. Tomato harvested in mature green stage was treated in water at 42℃ for 1 h. Samples of treated and non treated to mato harvested in mature green stage, as well as sample of non treated tomato harvested in red-ripe stage were stored at 4℃. Tomato ripening was monitored once a week by instrumental measurement of fruit color using Minolta Chroma Meter CR-400. After three weeks of cold storage, all tomato samples were evaluated by sensory panel consisting of 10 trained assessors. Differences between treated and non treated tomato samples were observed and compared with properties of red-ripe harvested tomato.
It was concluded that heat treatment in water of 42℃ for 1 h results in less chilling injuries, delayed but more uniform development of red color, less presence of vessels, and lower intensity of non-characteristic odor and taste.
Key words: *tomato, heat treatment, storage, ripening, color, sensory evaluation*

INTRODUCTION

Postharvest heat treatments are used for disinfestation and disinfection, modification of fruit response to temperature extremes and controlling of ripening process (Lurie, 1998, Paull and Chen, 2000). In the last decades, there has been increased interest for heat treatments partly because they can replace chemicals used against pathogens and insects (Lurie, 1998). Subtropical fruit, such as tomato (*Solanum lycopersicum* L.), develop chilling injury when stored at temperatures below 10-12℃ (Lurie and Klein, 1991, Sabehat et al., 1996). Chilling injury is physiological disorder whose symptoms are tissue browning, appearance of pits and discolorated areas on skin, non-uniform ripening and increased susceptibility to diseases (Luengwilai et al., 2012).
Heating induces many physiological changes in fruit. High temperature treatment (38℃ for 3 days) inhibited reversibly ethylene pro duction, color development and softening in tomatoes by decreasing expression of mRNAs related to ripening (Lurie et al., 1996). However, fruits held at low temperature (2℃ for 14 days) after heating ripened normally when placed at 20℃. Lurie et al. (1996) explained this by accumulation of heat-shock proteins (HSPs) caused by high temperature stress. It is supposed that HPSs stabilize structure and function of membrane proteins during and after heat treatment. This allows tomatoes to be stored at lower temperatures without developing of chilling injuries (Sabehat et al., 1996).
There are three methods for heating fruits: hot water, vapor heat and hot air. Vapor heat and hot air can be static or with high flow rate; in addition, hot air can have

humidity regulation. All these methods require different time of application in order to achieve desired effect on fruit (Lurie, 1998). Uniformity of heating is also an important factor for tomato ripening and chilling injury development. In research conducted by Lu et al. (2010), one half of tomato was exposed to heated air flow at temperature of 39℃ and velocity of 0,24 m/s, while the other half was held at room temperature (39℃) and same air flow velocity. Heated tomato halves were significantly different from unheated halves in terms of chilling injuries and color because process of postharvest ripening was delayed.

Different temperatures of hot water and treatment durations have been used for postharvest treatment of tomato. McDonald et al. (1999) found that treatment of tomatoes in water for 1 h at 42℃ reduced decay by 60%, when tomatoes were stored at 2℃ for 14 days before ripening at 20℃. Tomato fruits immersed in 40℃ water for 7 min showed reduced chilling injury symptoms (Luengwilai, 2012).

Postharvest heating of tomatoes in air at 34℃ for 24 h before storage at 4℃ or 10℃ for up to 4 weeks resulted in little heat injury and the least losses in antioxidant content, and fruit color developed normally (Yahia et al., 2007, Soto-Zamora et al., 2005).

The aim of this research was to compare sensory properties of heat treated tomatoes harvested at mature green stage with non treated mature green and red-ripe harvested tomatoes, after storage at 4℃ for 4 weeks. Changes in color values during cold storage were also determined.

MATERIAL AND METHODS

Heat treatment

Commercial tomato, grown in greenhouse, was harvested at mature green and red-ripe stage. Tomato harvested in mature green stage was treated in water at 42℃ for 1 h (McDonald et al., 1999). Samples of treated and non treated tomato harvested in mature green stage, as well as sample of non treated tomato harvested in red-ripe stage were stored at 4℃ for 4 weeks.

Color measurement

Tomato ripening was monitored on 0, 5, 14, 21 and 28 days of storage by instrumental measurement of fruit color using Chroma Meter CR-400 (Konica Minolta, Japan). The CIE L* (lightness), CIE a* (red-green) and CIE b* (yellow-blue), C* (chroma), h (hue angle), and DWL (dominant wavelenght) were read using a D_{65} light source and the observer angle at 2℃. Each sample was measured at six locations: two locations between the equator and the stem; two on the equatorial region, and two between the equator and the blossom end.

Sensory evaluation

Sensory profiling was performed by ten trained panellists between (35-50) years old. The assessors were selected from previously trained academic staff of the Institute of Food Technology, Novi Sad. Sensory evaluation was performed using a generic descriptive analysis technique, included 19 selected representative properties. The properties were evaluated using a 150 mm unstructured line scale (anchored at 15 mm and 135 mm with slight to very, respectively) (ISO, 2003). The anchored reference terminology definitions and evaluation techniques were agreed upon by the assessors during training (Pestorić, 2011). A total of two sessions was held during storage of samples. Samples of treated and non treated tomato harvested in breaker stage, as well as sample of non treated tomato harvested in red-ripe stage were presented to each assessor in each session providing a total of two replicate evaluations. All samples were presented to each assessor at the same time. The

order of sample presentation was completely randomized among assessors, indentified with three random numbers.

Data analysis

Results were expressed as the mean of replications for all measurements. Analysis of variance (ANOVA) and Duncan's multiple range test were used to compare means at 5% significance level by using the statistical data analysis software system STATISTICA (StatSoft, Inc. (2011), version 10.0 (www.statsoft.com)).

RESULTS AND DISCUSSION

Comparison of L*, a*, b*, C*, h, and DWL values of non treated, treated and red-ripe tomatoes is presented in Table 1. After 28 days of cold storage, L* values for neither non treated nor treated tomatoes reached value obtained by red-ripe tomato. On 21 and 28 day of storage, a* values for both non treated and treated tomatoes didn't differ significantly from that of red-ripe tomatoes. This can be explained by accumulation of lycopene in non treated and treated fruits in quantities similar to that in red ripe tomatoes, because increase of a* value is directly related to lycopene synthesis (Arias et al., 2000). The greatest increase of a* values can be seen between 5 and 14 days for treated tomatoes. Second to that is increase between 0 and 5 day of storage for non treated tomatoes. From these results, it can be concluded that ripening process is delayed in treated tomatoes in comparison to non treated tomatoes. Change of b* values cannot be connected with ripening in both samples. C* values for both non treated and treated tomatoes increased sharply between 5 and 14 day of storage and then remained constant. In contrast, hue angle values for both non treated and treated tomatoes decreased during storage period, and on 28 day there was no significant difference between them and red-ripe tomatoes. On 5 and 14 day of storage, significant difference between treated and control fruits can be observed. Similar results are obtained for dominant wavelenght in terms of change during storage. However, its values for both samples were significantly different from value for red-ripe tomato even on 28 day of storage.

Table 1. Comparison of color values of non treated, treated and red-ripe tomatoes

Treatment	Storage period [days]	L*	a*	b*	C*	h	DWL [nm]
Red-ripe	0	40,106^{a}	31,666^{a}	27,503^{cd}	42,004^{b}	41,013^{a}	598,264^{a}
Non treated	0	60,871^{g}	-8,314^{f}	25,947^{d}	27,311^{d}	107,572^{i}	570, 205^{h}
Treated	0	60,764^{g}	-6,462^{f}	26,021^{d}	26,918^{d}	103,839^{h}	571,549^{h}
Non treated	5	56,035^{e}	10,486^{d}	24,092^{e}	27,072^{d}	66,599^{f}	583,974^{f}
Treated	5	58,540^{f}	2,069^{e}	26,029^{d}	26,503^{d}	85,256^{g}	577,648^{g}
Non treated	14	47,705^{d}	26,366^{b}	32,010^{ab}	41,836^{b}	51,268^{de}	592, 087^{d}
Treated	14	48,985^{d}	22,946^{c}	30,809^{b}	38,680^{c}	53,711^{e}	590, 268^{e}
Non treated	21	44,867^{c}	30,080^{a}	33,535^{a}	45,256^{a}	48,294^{cd}	594,203^{c}
Treated	21	44,985^{c}	30,128^{a}	32,123^{ab}	44,236^{a}	46,912^{bc}	594,709^{bc}
Non treated	28	42,784^{b}	30,003^{a}	27,503^{c}	41,736^{b}	43,836^{ab}	596,197^{b}
Treated	28	44,223^{bc}	31,278^{a}	31,030^{b}	44,256^{a}	44,783^{abc}	595,967^{b}

Values are means of thirty measurements.
Values with the different superscript within a column are statistically different (P < 0.05).

Concerning the sensory properties (Table 2), non treated and treated tomatoes stored for 21 day at 4℃ were significantly differe nt from red-ripe tomatoes in terms

of: fruit red color intensity, fruit color uniformity, firmness, juice leakage, fruit compactness and juiciness. In research conducted by Auerswald et al. (1999), properties that changed most during postharvest period were, among others, red color of tomato surface, color intensity of fruit flesh, firmness (finger touch) and juiciness. In our research, all samples differ significantly from each other in terms of cross-section red color intensity, with highest scores for red-ripe and lowest for non treated tomatoes. Cross-section red color uniformity was significantly lower for non treated tomatoes. Non treated tomatoes had significantly higher scores for vessels, off-odor intensity and off-taste intensity. There were no significant differences in terms of cross-section cavities, skin and pulp chewiness, sweetness, sourness and taste intensity between evaluated tomato samples.

Table 2. Sensory evaluation of tomatoes at 21 day of storage

Sensory property	Red ripe	Non treated	Treated
Fruit red color intensity	64,86^{a}	36,14^{b}	39,14^{b}
Fruit color uniformity	112,14^{a}	50,71^{b}	40,71^{b}
Firmness	74,29^{a}	127,14^{b}	120,29^{b}
Juice leakage	58,57^{a}	9,14^{b}	5,14^{b}
Cross-section red color intensity	55,57^{a}	22,57^{c}	40,86^{b}
Cross-section color uniformity	84,57^{a}	21,43^{b}	100,43^{a}
Vessels	5,29^{a}	54,86^{b}	5,86^{a}
Fruit compactness	107,14^{a}	130,29^{b}	133,42^{b}
Odor intensity	98,29^{a}	48,86^{b}	75,71^{ab}
Off-odor intensity	1,57^{a}	17,86^{b}	1,43^{a}
Cross-section cavities	1,86^{a}	2,43^{a}	1,86^{a}
Skin chewiness	122,42^{a}	124,43^{a}	98,14^{a}
Pulp chewiness	51,57^{a}	75,14^{a}	61,14^{a}
Juiciness	95,86^{a}	38,29^{b}	39,57^{b}
Granularity	57,43^{a}	86,86^{ab}	100,57^{b}
Sweetness	30,14^{a}	32,00^{a}	26,00^{a}
Sourness	78,00^{a}	87,57^{a}	101,43^{a}
Taste intensity	71,29^{a}	50,86^{a}	57,29^{a}
Off-taste intensity	0,71^{a}	16,14^{b}	2,57^{a}

Values are means of two measurements.
Values with the different superscript within a row are statistically different (P < 0.05).

CONCLUSIONS

Heat treatment in water increased cross-section red color intensity and color uniformity, and also caused less presence of vessels, as well as lower intensity of off-odor and off-taste. It also induced delay of ripening in treated tomatoes in comparison with non treated samples.

ACKNOWLEDGEMENTS

This paper is a result of the researches within the Project No. III 46001 financed by the The Ministry of Education and Science, Republic of Serbia.

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INFLUENCE OF HYDROCOLLOIDS ON THE RHEOLOGICAL PROPERTIES OF BUCKWHEAT DOUGH

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ABSTRACT: Due to the greater awareness among the scientists and technologists regarding unbalanced diet of celiac patients, buckwheat flour has been recently in a focus as a novel material in gluten-free formulations because of its unique nutritional profile. However, the use of buckwheat flour in baked product formulations has been limited due to the lack of gluten-forming protein which imparts the dough viscoelastic properties and gas retention. In order to improve the processing properties of gluten-free cereals and pseudocereals hydrocolloids are commonly used as gluten substitutes.
Therefore, the aim of the present study was to investigate the influence of different hydrocolloids (xanthan and hydroxypropyl methylcellulose) on thermomechanical and viscoelastic properties of buckwheat dough. Concerning the thermomechanical behaviour determined by Mixolab, xanthan and hydroxypropyl methylcellulose incorporation in buckwheat dough resulted in significantly decreased water absorption and increased dough development time. Moreover, due to the specific behaviour of examined systems, standard Mixolab protocol was modified by increasing the dough weight from 75 to 90 g and duration of dough mixing during the cold stage from 8 to 15 min. During the heating stage, the addition of xanthan significantly affected the weakening of the buckwheat dough induced by mixing and temperature enhancement, as well as the peak viscosity, while hydroxypropyl methylcellulose significantly affected breakdown torque. The study of rheological properties of dough by oscillatory and creep measurements showed that xanthan significantly strengthened the buckwheat dough.
Key words: *hydrocolloids, buckwheat dough, Mixolab, rheology*

INTRODUCTION

Coeliac disease may be defined as a long-life intolerance to wheat gliadin fraction and rye prolamins (secalins), barley (hordeins) and possibly oats (avidins) (Murray, 1999). Since gluten is the main structure-forming protein in flour which is responsible for the elastic properties of dough, its removal in gluten-free products represents major challenge to the cereal technologist (Gallagher et al., 2004). Lack of viscoelasticity of different gluten-free dough might be overcome by the addition of different gums and hydrocolloids which represents essential ingredient in gluten-free formulations for texture and appearance improvement of the final product. The incorporation of gums and hydrocolloids assures higher dough consistency, improved gas retaining properties and longer shelf life due to their structure forming ability (Mariotti et al., 2009). According to Thompson (1999) gluten-free food formulation are frequently produced from refined gluten-free flour or starch and they are generally not enriched or fortified (Thompson, 1999). Therefore, there is a rising trend in the addition of different ingredients with improved nutrient profile in gluten-free formulation. Due to its high content of polyphenol/flavonoids compounds, minerals,

essential amino acids (Mariotti et al, 2008; Morita et al., 2006) etc., buckwheat flour is gaining more popularity in gluten-free formulations.
Since influence of different hydrocolloids on rheological properties of wheat flour, rice flour and gluten-free mixtures was already investigated, the aim of the present study was to investigate the effect of xanthan and hydroxypropyl methylcellulose on thermomechanical and viscoelastic properties of buckwheat dough.

MATERIAL AND METHODS

Wholegrain buckwheat flour, BF (11.1% moisture) was purchased from a local market. The hydrocolloids used in this study were hydroxypropyl methylcellulose, HPMC (Alfa Aesar, Germany) and xanthan (Roth, Germany) which were added to buckwheat dough at 1.5%.
The effect of hydrocolloids on dough thermomechanical properties was investigated using Chopin Mixolab (Tripette et Renaud, Paris, France) with the modified Chopin+ protocol. The parameters of the protocol are listed in Table 1.

Table 1. Modified Chopin+ Mixolab parameters

Setting	Values
Mixing speed	80 rpm
Target torque (for C1)	1.1 Nm
Dough weight	90 g
Tank temperature	30 ℃
Temperature first step	30 ℃
Duration first step	15 min
Heating rate	4 ℃/min (15 min)
Temperature second step	90 ℃
Duration second step	7 min
Cooling rate	4 ℃/min (10 min)
Temperature third step	50 ℃
Duration third step	5 min
Total analysis time	52 min

Dough samples for the rheological tests were prepared using Mixolab bowl, at 30 ℃. The amount of added water corresponded to Mixolab water absorption, on 14% moisture basis. Total mixing time was 15 min. For the rheological measurements of buckwheat dough, Haake Mars rheometer (Thermo Scientific, Germany), operated at 30 ℃ and equipped with two 35 mm serrated plates was used. The buckwheat dough was placed between plates within 10 min after mixing. The excess edges of the sample were trimmed; the rim of the sample was coated with paraffin oil and the test was started after the dough was rested for 10 min. The tests performed on the dough samples were: a) frequency sweep test from 0.1 to 10 Hz under a 5 Pa stress, which was within the linear viscoelastic region and b) creep test at constant shear stress of 200 Pa for 300 s, followed by strain recovery in 600 s after removal of load.

RESULTS AND DISCUSSION

The effect of xanthan and hydroxypropyl methylcellulose on the thermomechanical properties of buckwheat flour is shown in Figure 1. While the first stage (from the beginning to C2 value) mainly described the properties of BF proteins, the second stage (from C2 to the end of the profile) reflected the behaviour of starch component.

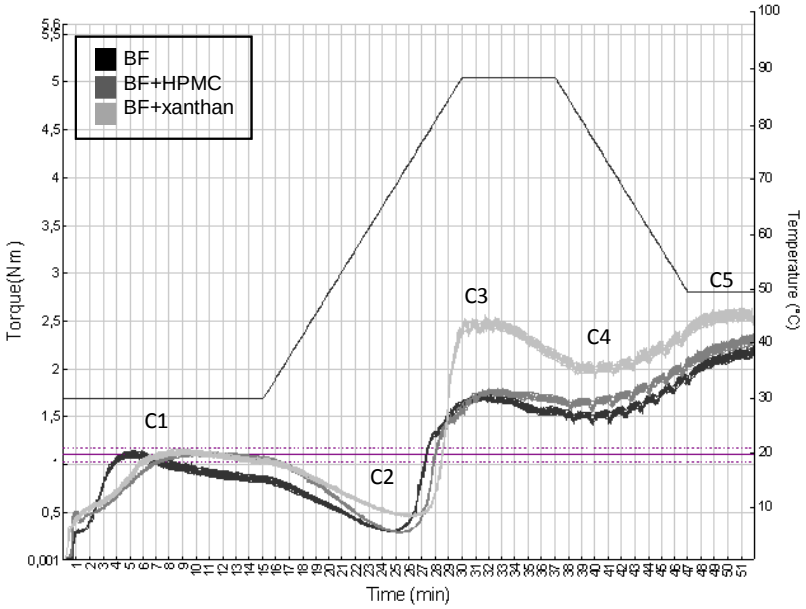

Figure 1. Effect of hydrocolloids addition on mixolab profile of buckwheat dough

In contrast to wheat flour (Rosell et al., 2007) and gluten-free flour blends (Lazaridou et al., 2007), water absorption of buckwheat flour decreased with the hydrocolloids addition. Wholegrain buckwheat flour exhibited higher water absorption (WA = 67.3%) than wheat flour (Rosell et al., 2007) and the presence of HPMC and xanthan lowered the WA values to 65.3 and 62%, respectively. Due to the ability of hydrocolloids to slowly hydrate, the dough development time of buckwheat flour was greatly increased in the presence of hydrocolloids, resulting in an increase of 93% for HPMC and 76% for xanthan. Due to the specific behaviour of examined systems, standard Mixolab Chopin+ protocol was modified by increasing the dough weight from 75 to 90 g and duration of dough mixing during the cold stage from 8 to 15 min (Table 1). According to results obtained by Rosell et al. (2007) who examined the influence of HPMC, pectin, guar and xanthan addition on thermomechanical properties of wheat flour, HPMC was the polymer with the strongest effect on dough development time and dough stability. Similar results had also been reported by Sivaramakrishnan et al. (2004) in the case of rice flour fortified with 4.5% HPMC. As can be observed in Figure 1, the addition of xanthan strengthened the buckwheat dough, as revealed by increase in C2 value, which reflects the dough resistance to mechanical and thermal stress. On contrary, HPMC did not affect the C2 parameter value. Except buckwheat protein weakening and denaturation, further increase in temperature (from 59 – 60.8 ℃ to 90 ℃) led to sta rch granule swelling, disintegration and leaching of amylose and amylopectin. C3 is the parameter which indicates starch gelatinization (Ozturk et al., 2008). The value of peak torque, C3, was greatly increased by xanthan incorporation, while hydroxypropyl methylcellulose decreased breakdown torque (difference between C3 and C4 value). On contrary, setback torque (difference between C5 and C4 value) which refers to starch retrogradation was not affected by hydrocolloids addition.

The effect of hydrocolloids on viscoelastic properties of buckwheat dough was also investigated. Mechanical spectra (Figure 2) of all samples showed that the value of storage modulus (G') increased with an increase in frequency, while loss modulus (G") was nearly independent of frequency. This characteristic of buckwheat dough was also reported by Han et al. (2011).

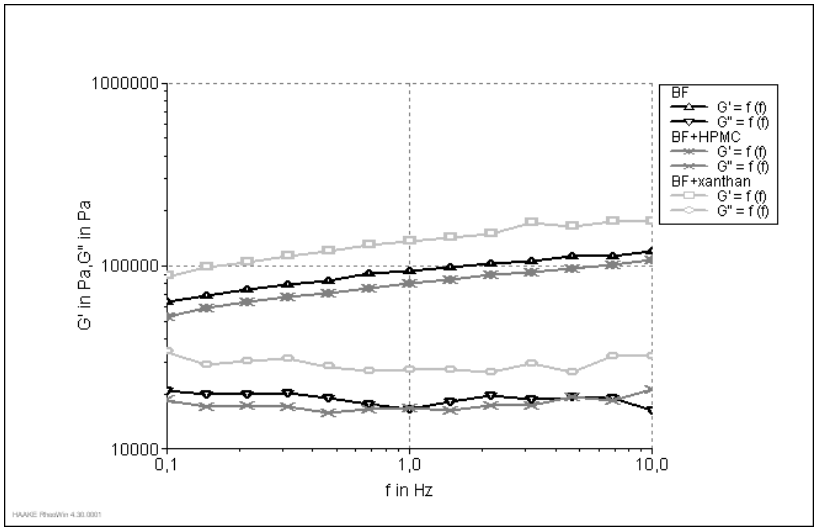

Figure 2. Effect of hydrocolloids addition on mechanical spectra of buckwheat dough

In general, the value of G' was higher than G" (tan δ < 1) in the whole range of frequencies which indicated a prevalence of elastic properties over viscous. The highest dough strength, as revealed by G' value, was observed in xanthan, following by control sample (pure buckwheat dough) and the lowest in HPMC containing sample.

Creep and recovery curves of the control dough and samples with the addition of hydrocolloids are shown in Figure 3. The lowest value of maximum strain, i.e. the highest resistance to deformation was obtained for sample which contained xanthan, while the lowest resistance to deformation was observed in samples with HPMC. Therefore, it can be concluded that parameters which are related to dough strength (C2 value in Mixolab, G' value in frequency sweep test and maximum strain value in creep and recovery test) were in accordance.

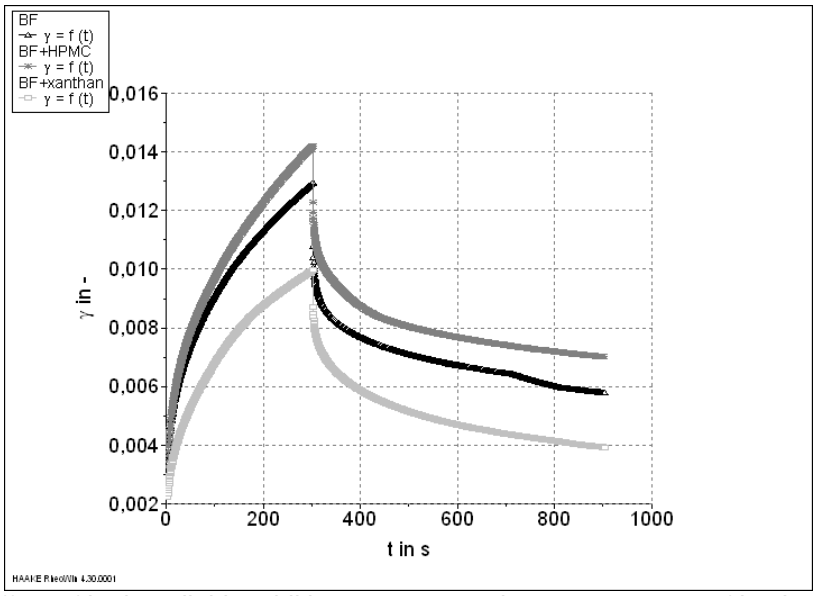

Figure 3. Effect of hydrocolloids addition on creep and recovery curves of buckwheat dough

In the recovery phase, approximately 60% elastic recovery could be seen for xanthan containing dough, while the recovered deformation for HPMC and control dough was 51 and 55%, respectively.

The ability of xanthan to enhance dough strength and elasticity was reported earlier for gluten-free flour blend (Lazaridou et al., 2007) and ascribed to its rigid, ordered chain conformation and high viscosity of its solution.
On contrary, HPMC addition slightly lowered buckwheat dough rigidity and elasticity. According to literature findings, opposite conclusions concerning the influence of HPMC have been reported. Sivaramakrishnan et al. (2004) have revealed that high viscous character has been imparted by HPMC addition to rice flour. On contrary, Lazaridou et al. (2007) have reported that the addition of HPMC into gluten-free flour blend resulted in creep and recovery curves which shifted to higher values.

CONCLUSIONS

It can be concluded that influence of the HPMC and xanthan on thermomechanical and viscoelastic properties of buckwheat dough greatly depend on the nature of added hydrocolloid. While the incorporation of HPMC mainly affected dough development time and only slightly decreased dough strength, xanthan caused greater effects on buckwheat dough, which was expressed as increase in dough strength and elasticity as well as rise in maximum viscosity during heating. Moreover, in buckwheat flour, which already contains natural hydrocolloids in contrast to wheat and rice flour, the changes induced with further hydrocolloid addition are somewhat different than the one observed in other flours.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31007 - Evaluation of quality and optimisation of processing of wheat affected by climatic changes (Vrednovanje kvaliteta i optimizacija prerade pšenice u svetlu klimatskih promena), financed by the Ministry of Education and Science, Republic of Serbia.

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INFLUENCE OF TWISTED TAPE AND BLADE TYPE TURBULENCE PROMOTERS ON THE MICROFILTRATION OF MILK

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ABSTRACT: The aim of this research was to study influence of turbulence promoters on permeate flux improvement and energy savings during microfiltration of milk. Low pressure loss turbulence promoters, twisted tape and blade shaped static mixer, named Koflo, were used. The chosen turbulence promoters are of the same aspect ratio (ratio of pitch length to diameter of promoter) 2.5 and inserted into the tubular ceramic membrane (0.1 μm pore size). By applying turbulence promoters, fouling was reduced and the permeate fluxes were increased by a factor of three for twisted tape and by a factor of four for blade shaped static mixer. Intensity of turbulence in a module fitted with promoter depends on the applied cross flow rate. An increase of cross flow rate induces a rise of flux in the both modes of operation, with and without turbulence promoters. When compared at the same cross flow rate, the flux obtained by application of turbulence promoters is always higher than the flux obtained without promoters. The cross flow rates, under which the maximal flux improvement was achieved (300% for twisted tape and 320% for Koflo), are several folds lower than that applied in operation without promoter. In the presence of turbulence promoters permeate flux was increased as well as the pressure drop along membrane. Despite the higher pressure drop, operation with turbulence promoters provides significantly reduced energy consumption compared to operation without turbulence promoters.
Keywords: *turbulence promoters, twisted tape, blade shape static mixer, microfiltration*

INTRODUCTION

Pressure driven membrane processes are widely used in the food industry to substitute conventional concentration, separation and clarification techniques. Applications of membrane filtration in the dairy industry include bacterial removal, selective separation of micellar casein and milk fat, fat removal from whey by microfiltration (MF), milk concentration by ultrafiltration, cheese or milk permeate concentration by nanofiltration [Saboya, Maubois, 2000]. The efficiency of a pressure driven membrane process essentially depends on maintenance the stable permeate flux. Permeate flux value is limited by membrane fouling [Palacio at al., 2003]. During microfiltration, membrane fouling can lead to more than an order of magnitude reduction in the filtrate flux and this fouling is typically caused by the deposition of large protein aggregates on the membrane surface [Bowen, Gan, 1991; Belfort at al., 1994]. Both protein adsorption and protein–protein interactions can narrow or plug pores hence increasing the rejection of permeate particles. Rejected particles can be accumulated at the surface of the membrane due to their slow diffusion back into the retentate, which can be reduced, but not eliminated by cross-flow filtration. Accumulation of particles causes concentration gradient at the membrane surface resulting in the fouling mechanism known as concentration polarization [James et al., 2003].
Physicochemical properties of the solute and the hydrodynamics of the membrane system also have significant effects on membrane fouling. The rate and extent of irreversible membrane fouling depend on the membrane material and operating parameters (transmembrane pressure, crossflow velocity, temperature) [Jones, O`Melia, 2001]. Chemical, mechanical and hydrodynamic methods and/or pre-treatment are used to reduce fouling [Brans et al., 2004]. Also, in order to reduce

fouling, different techniques such are vibrations, backflushing, backpulsing, rotations, have been developed [Fuente et al., 2010; Sondhi, Bhave, 2001; Williams, Wakeman, 2002]. One of the hydrodynamic techniques for fouling reduction is an increase of turbulence intensity by the introduction of motionless turbulence promoters in the flow channel. The insertion of turbulence promoters in the membrane module is proved to be efficient technique from the point of view of energy consumption, capital costs and maintenance, while the achieved flux improvement can be above 300% depending on the filtered system and applied operating conditions [Krstić et al., 2003, 2007]. Hydrodynamics in module can be changed using different types of turbulence promoters such as KM Kenics^{TM} static mixers, helical screw-thread, cone shape inserts, metal grills, spiral wire, disc and doughnut shape inserts, twisted tapes and smooth rods.
The objective of this study was to investigate influence of turbulence promoters geometry on the permeate flux improvement in the MF process. In this work, twisted tape and blade shaped static mixer, named Koflo^{TM}, were chosen because they cause low pressure drop along the membrane. The effects of these two turbulence promoters, with different geometry and same aspect ratio of 2.5, on permeate flux improvement are analysed. Also, energy consumption using the turbulence promoters was investigated in this work.

MATERIALS AND METHODS

Pasteurized and homogenized (one stage, 150bars), partially skimmed milk was used as feed throughout the cross-flow microfiltration experiments. The composition of fresh milk (%, w/w) was as follows: 3.1 proteins, 2.8 fat, 3.5 lactose and 0.7 ashes (Dukat Dairy Industry, Serbia). The pH of milk was 6.8 ± 0.15 at 50 ◦C. The particle size distribution in milk was measured using a Malvern Nano ZS analyser at 20 ◦C (Malvern Instruments Ltd., Worcestershire, United Kingdom). For the particles in milk, an average hydrodynamic diameter of 185 ± 8.0 nm was determined.
All experiments were performed using the laboratory scale microfiltration/ ultrafiltration experimental unit made of stainless steel. Experiments were performed using a single channel ceramic membrane, 250 mm long, with 7 mm ID and 10 mm OD (GEA, Germany). The 0.1 µm membrane made of a zirconium filtering layer on an α-alumina support. Active filtering area of membrane was 46.2 cm^2. Permeate was collected in the container placed on the digital balance (EW 1500-2 M, KERN Germany) and continuously weighted while the data were transferred to personal computer.
Each experiment included the following steps: pure water flux measurement, microfiltration, rinsing, chemical cleaning, rinsing, and pure water flux measurement. The feed flow rate was varied from 1.0 to 6.0 L min^{-1} while the transmembrane pressure was 50 kPa in all of the tests. All experiments were carried out at 50 ± 0.5 ^{o}C.
Experiments were carried out with and without turbulence promoters in order to examine effects of turbulence promoters on microfiltration process. As turbulence promoters the twisted tape and Koflo static mixer were used (Fig.1). For turbulence promoters the main geometrical characteristic is an aspect ratio (O_{TP}) defined as ratio of the pitch length to the diameter. Turbulence promoters were manufactured in the workshop Inox Bravarija (Bački Petrovac, Serbia) and their overall characteristics are given in Table 1.

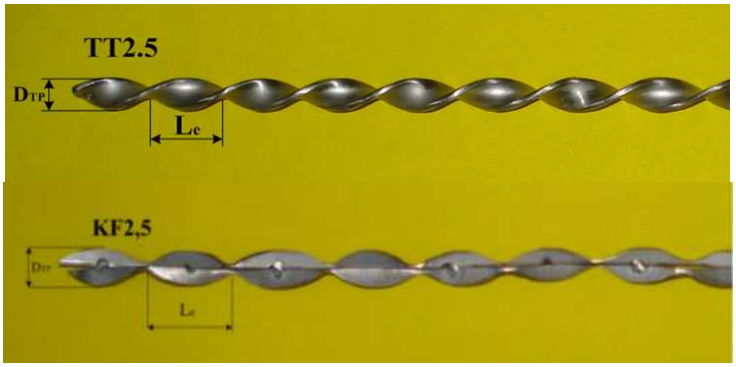

Fig. 1. Turbulence promoters

Table 1. Characteristic of twisted tape and Koflo static mixer with aspect ratio of 2.5

	TT2,5	KF2,5
Material	Stainless still	
D_{TP} (mm)	6,3	6,2
L_{TP} (mm)	243	250
Le (mm)	15.5	15.6
N_{TP}	16	16
O_{TP}	2,5	2,5

To evaluate the efficiency of turbulence promoters two references are chosen: flux improvement (FI) and specific energy consumption (E). The FI represents a relative comparison of fluxes obtained with and without a turbulence promoters application, as follows:

$$FI = \frac{J_{TP} - J_{NTP}}{J_{NTP}} \times 100\% \quad (1)$$

The specific energy consumption (E) represents the hydraulic power dissipated (HDP) per unit volume of permeate. The HDP depends on the pressure loss (ΔP) along the membrane module and the applied cross flow rate (Q), so the specific energy consumption is calculated from:

$$E = \frac{Q\Delta P}{J_p A_{ac}} \quad (2)$$

where A_{ac} is active surface of a membrane (m^2) and J_p is permeate flux ($Lm^{-2}hr^{-1}$).

RESULTS AND DISCUSSION

Comparison under constant cross flow rate

Permeate fluxes recorded during microfiltration of milk under the cross flow rate of 3 L min^{-1} and transmembrane pressure of 50 kPa is shown at Figure 2. The use of turbulence promoters, twisted tape (TT2.5) and Koflo (KF2.5) provided a significant flux enhancement compared to the operation without turbulence promoters (NTP). Namely, application of the twisted tape enables increase of the permeate flux from 17 to 55.5$Lm^{-2}hr^{-1}$ while Koflo static mixer enables increase from 17 to 75$Lm^{-2}hr^{-1}$.

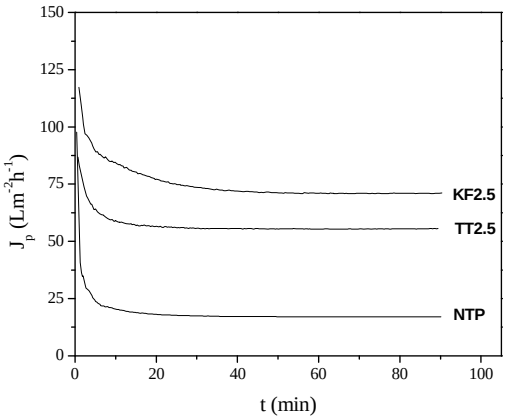

Fig.2 Time dependency of flux during milk microfiltration

The curves of permeate flux have the shape which indicates type of fouling typical for the microfiltration of milk (Popović, Tekić, 2011). The sharp flux decline is usually attributed to the concentration polarization and gel layer formation at the membrane surface. Application of turbulence promoters contributes to fouling reduction. In the presence of turbulence promoters, fluid acceleration occurs due to reduction in cross-sectional area of membrane. The local acceleration of fluid nearby the membrane surface reduces the formation of the boundary layer. This contributes to reduction of concentration polarization and consequently results in a high permeate flux.

Influence of cross flow rate

The permeate flux variation with cross flow rate for both NTP and turbulence promoters operation is shown in Fig. 3. As obvious, the much higher fluxes were obtained at lower flow rates by using turbulence promoters.

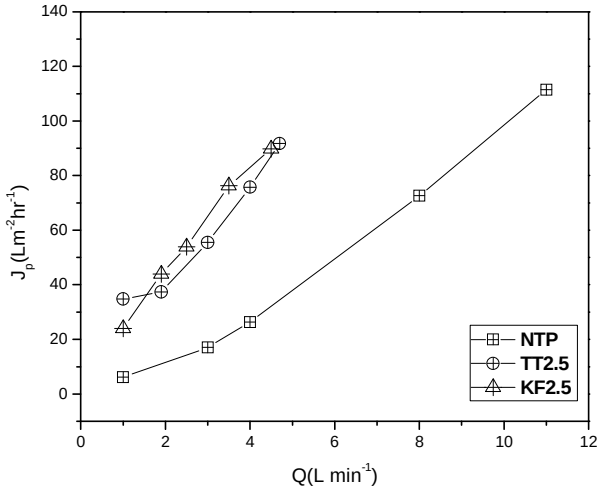

Fig.3. Permeate flux as a function of the cross flow rate

At the same cross flow rate, higher flux has been achieved using TT2.5 and KF2.5 compared to membrane without turbulence promoters (NTP). Further, when comparing values of permeate fluxes achieved with TT2.5 and KF2.5, it can be seen that KF2.5 gives higher permeate flux.

Change of hydrodynamic regime is not the only factor that is responsible for flux improvement. The most important factor in flux improvement is change in streamline path. In the presence of turbulence promoters, hydrodynamic regime in the

membrane is changed using only the energy of a moving fluid. The helical geometry of the twisted tape elements results in a change of axial flow to the helical flow. Helical path of the streamlines and the appearance of local vortices reduce the effects of concentration polarization and deposition of particles and results in a higher permeate flux. In the presence of Koflo static mixer, center of the membrane is blocked periodically in places where the blades intersect. Koflo divides fluid into two parts after each blade. A part of the fluid is forced to move helically along the wall membrane and reducing deposition of large proteins at the membrane surface.
The increase of the cross flow rate in NTP operation never resulted in such increase of the flux as it can be obtained by application of a turbulence promoters.

Efficiency of turbulence promoters
In order to completely prove the efficiency of turbulence promoters the values of flux improvement and energy consumption were analyzed. Fig. 4 shows the flux improvement obtained by application of turbulence promoters under the investigated range of cross-flow rates. The flux improvement of 180–300% is achieved by applying the TT2.5 while the flux improvement of 200–320% is achieved using KF2.5 static mixer. The cross-flow rate under which the maximal flux improvement can be achieved is about 2 L min^{-1} for twisted tape and about 1,5 L min^{-1} for Koflo static mixer.

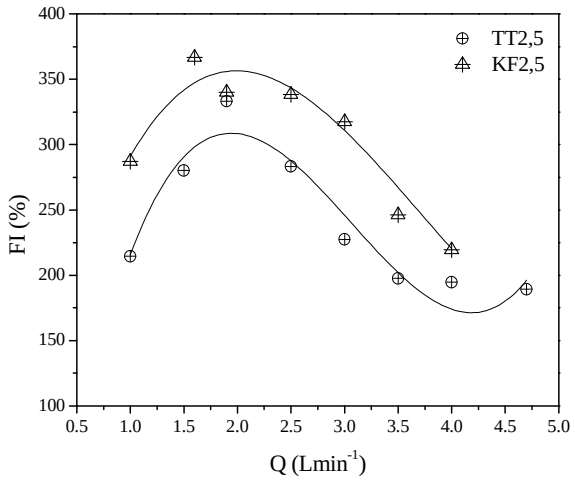

Fig. 4. Flux improvement as a function of the cross-flow rate.

In the presence of turbulence promoters permeate flux and pressure drop along membrane are increased. Pressure drop influences on the choice of transmembrane pressure and economic viability of microfiltration. With regard to empty membrane, turbulence promoters cause higher pressure drop and increase of power dissipation. To demonstrate that they are still efficient, the flux improvement for the same hydraulic dissipated power is presented in the Table 2.

Table 2. Flux improvement as function of the hydraulic dissipated power.

HDP (W)	J_p ($Lm^{-2}h^{-1}$)			FI (%)	
	NTP	KF2,5	TT2,5	KF2,5	TT2,5
0,08	12	24	20	100	67
0,25	29	44	43	52	48
0,4	35	51	56	54	70
1,0	52	74	78	42	50
2,0	73	86	92	19	26

The flux improvement of 19-100% was achieved by applying KF2.5 and improvement of 26-67% by applying TT2.5. This improvement decreases with an increase of the hydraulic dissipated power for turbulence promoters.
Despite the higher pressure drop, application of turbulence promoters enables significant reduction of energy consumption in the range of 45-67% for the twisted tape and 23-67% for the Koflo static mixer.

CONCLUSION

The results of experiments clearly show that application of turbulence promoters contributes to fouling reduction and improvement of permeate flux compared to results achieved in conventional microfiltration of milk. Permeate flux improvements of 300% for twisted tape and of 320% for Koflo static mixer were obtained at the same cross flow rate. The flux improvement using turbulence promoters was obvious also under the same hydraulic dissipated power indicating that an energy saving is possible using the turbulence promoters compared to operation without the promoters.

ACKNOWLEDGEMENT

The financial suport of the Ministry of Education and Science of the Republic of Serbia (grant number E!5406) is gratefully acknowledged.

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MATHEMATICAL MODELLING OF SOLAR DRYING OF UNPROCESSED APRICOT AND SULPHURATED APRICOT IN NATURAL CONVECTION SOLAR FOOD DRYER

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ABSTRACT: This paper presents a study on the mathematical modelling of solar drying of unprocessed apricot and sulphurated apricot. In this purpose, natural convection solar food dryer which has solar air collector was designed and manufactured for drying food products. Solar drying experiments of apricots were conducted in Izmir, Turkey and experiments were realized in two steps. In the first experiment, natural drying was done without applying any pretreatment. In the second experiment, apricots were dried after sulfuring pretreatment. During the drying period, relative humidity, air flow rates, drying air temperature, solar radiation and lose of food product's mass were measured continuously in the different parts of the dryer. Drying time is examined with mass ratio for each apricot type as polynomial and exponential correlations, separately. Fourteen different mathematical drying models available in literature were compared based on their coefficient of determination, mean bias error, root mean square error, modelling efficiency and reduced chi-square to estimate the solar drying curves. According to statistical analysis results, the Midilli and Kucuk drying model has shown a better fit to the experimental drying data of untreated apricot and sulphurated apricot with a coefficient of determination R^2 of 0, 9982 and 0, 9948, respectively.
Keywords: *mathematical modelling, apricot, sulphated apricot, solar food drying, mass ratio.*

INTRODUCTION

Turkey has a high potential solar enegy and means annual bright sunshine hours. According to experimental data, annual mean sunshine hours is 2640 hours/year (7.2 hours/day), annual mean solar energy is 1311 kWh/m^{2}year (3.6 kWh/m^{2}day) for Turkey (EIE, 2006). Many different types of vegetables and fruits have been produced in Turkey because of its suitable climate. Drying has always been of great importance for conserving agricultural products in agricultural countries like Turkey. Drying process is the most common form of food preservation and extends the food self-life. It is a simultaneous heat and mass transfer operation in which moisture is removed from food material and carried away by hot air. Open sun drying is a well-known food preservation technique that is still the most common method used to preserve agricultural product in most tropical and subtropical countries.
In Turkey, drying is achieved method by spreading out the material on the ground. In this way, there are many disadvantages like low quality and hygienic problems. The resulting loss of food quality in the dried products may have effect negatively trade potential and economical worth. For preventing the deterioration of the materials different types of drying methods have been developed. On the other hand, the conventional dryers are not economic due to high energy cost. For that reason, direct or indirect sun dryers have good opportunity for about quality and efficiency improvement. In this purpose, this study was undertaken to investigate drying characteristics of unprocessed apricot and sulphurated apricot in a solar dryer in Izmir, and to fit the experimental data to mathematical models available in literature.

MATERIAL AND METHODS

In this experimental study, solar dryer who has the dimensions of the drying unit are 1 m x 1 m x 1.1 m is used for drying of apricot. This solar dryer consists of a flat plate solar air collector and drying unit as shown in Figure 1. The dryer and solar air collector was oriented in an east-west direction to make the incident solar radiation more efficient on the solar dryer. Drying unit was covered with polyethylene plastic film material. Three drying trays, having dimensions of 1 m x 1 m, were placed in the solar dryer. These trays were used to accommodate apricots to be dried. The solar air collector dimensions were 1 m wide by 2 m long and 0.17 m high. A corrugated galvanized iron sheet painted black was used as an absorber plate for absorbing the solar radiation. A polyethylene plastic film material was used as a transparent cover for the air collector to prevent the heat losses. Styrofoam is used as insulation material to prevent heat losses from the sides and bottom of solar collector. Heat is transferred from the absorber surface to the air in the collector and heated air while passing over the product absorbs moisture.

Figure 1. View of solar dryer

Drying experiments were conducted in Izmir and experiments were realized in two steps. In the first experiment, natural drying was done without applying any pretreatment. In the second experiment, apricots were dried after sulfuring pretreatment. The important parameters as drying air temperature, relative humudity, air flow rates, solar radiation, and lose of mass affecting the performance of the dryer were measured. A pyranometer was used to measured the solar radiation on the horizontal surface. The temperature and relative humidity inside and outside of the dryer were measured with a temperature and relative humidity probes. The velocity of drying air was measured with an anemometer at the inlet of the dryer.

Mathematical Modeling of Solar Drying Curves

The drying curves obtained were fitted with fourteen different moisture ratio equations by several authors in Table 1 (Diamente et al., 1993; Sharaf-Elden et al., 1980; Togrul and Pehlivan, 2002; Liu and Bakker-Arkema, 1997; Karathanos and Belessiotis, 1999; Koua et al., 2009; Goyal et al, 2006; Gungor and Ozbalta, 2003; Tunde-Akintunde, 2011; Vega-Galvez, 2010; Midilli et al., 2002; Gurlek et al., 2009). The moisture ratio (MR) was simplified to M_t/M_o as written in the following table and M_o is sample mass at the beginning and M_t is sample mass in time t.

Table 1. Mathematical models given by various authors for the solar drying curves

Model	Model equation	Model name
1	$(M_t / M_o) = \exp(-kt)$	Newton
2	$(M_t / M_o) = \exp(-kt^n)$	Page
3	$(M_t / M_o) = \exp(-(kt)^n)$	Modified Page
4	$(M_t / M_o) = \exp(-k(t/l^2)^n)$	Modified Page Equation
5	$(M_t / M_o) = a \exp(-kt)$	Henderson and Pabis
6	$(M_t / M_o) = a \exp(-kt) + b \exp(-gt) + c \exp(-ht)$	Modified Henderson and Pabis
7	$(M_t / M_o) = a \exp(-kt) + c$	Logarithmic
8	$(M_t / M_o) = a \exp(-k_1t) + b \exp(-k_2t)$	Two-Term
9	$(M_t / M_o) = a \exp(-kt) + (1-a) \exp(-kat)$	Two-Term Exponential
10	$(M_t / M_o) = 1 + at + bt^2$	Wang and Singh
11	$(M_t / M_o) = a \exp(-kt) + (1-a) \exp(-gt)$	Verma et al.
12	$(M_t / M_o) = a \exp(-kt) + (1-a) \exp(-kbt)$	Diffusion approximation
13	$(M_t / M_o) = a\exp(-kt^n)+bt$	Midilli and Kucuk
14	$(M_t / M_o) = at^3+bt^2+ct+1$	Authors approximation

The experimental drying data for unprocessed apricot and sulphurated apricot were fitted to drying models in Table 1 by using regression analysis. The coefficient of determination (R^2) was one of the important criteria to select the best equation in the solar drying curves of the dried samples as apricot. In addition to R^2, the various statistical parameters such as; reduced Chi-square ($\chi 2$), mean bias error (MBE), the root mean square error (RMSE) and the modelling efficiency (EF) were used to determine the quality of the fit. The lower the values of the $\chi 2$, the better the goodness of the fit. The RMSE gives the deviation between the predicted and experimental values and it is required to reach zero. The EF also gives the ability of the model and its highest value is 1 (Gurlek et al., 2009). These parameters can be calculated as;

$$\chi^2 = \frac{\sum_{i-1}^{N} \left(MR_{\exp,i} - MR_{pre,i}\right)^2}{N-n}$$

$$MBE = \frac{1}{N}\sum_{i-1}^{N}\left(MR_{pre,i} - MR_{\exp,i}\right)$$

$$RMSE = \sqrt{\frac{\sum_{i-1}^{N}\left(MR_{pre,i} - MR_{\exp,i}\right)^2}{N}}$$

$$EF = \frac{\sum_{i=1}^{N}\left(MR_{\exp,i} - MR_{i,\exp mean}\right)^2 - \sum_{i=1}^{N}\left(MR_{pre,i} - MR_{\exp,i}\right)^2}{\sum_{i=1}^{N}\left(MR_{\exp,i} - MR_{i,\exp mean}\right)^2}$$

where $MR_{exp,i}$ is the experimental moisture ratio found in any measurement and $MR_{pre,i}$ is predicted moisture ratio for this measurement. N the number of observations, n the number of constants and $MR_{expmean}$ is the mean value of the experimental MR. The best model describing the drying behaviour of apricot was chosen as the one with the highest coefficient of determination and the modelling efficiency and the least reduced Chi-square, mean bias error and root mean square error (Gurlek et al., 2009).

RESULTS AND DISCUSSION

In this study, the constant and coefficients of the best fitting mathematical model involving the drying variables such as temperature, humidity of the drying air were determined. The changes in the moisture ratio of natural drying of apricot with drying time are shown in Figure 2. The experimental data obtained for air at temperature

ranging from 48 ^{0}C to 57.1 ^{0}C. The interruptions of the lines in this figure represent the night periods of the drying operation. The drying continues after the sunset due to the thermal inertia of the system. There is not any constant-rate drying period in this curve and all the drying operations are seen to occur in the falling rate period.

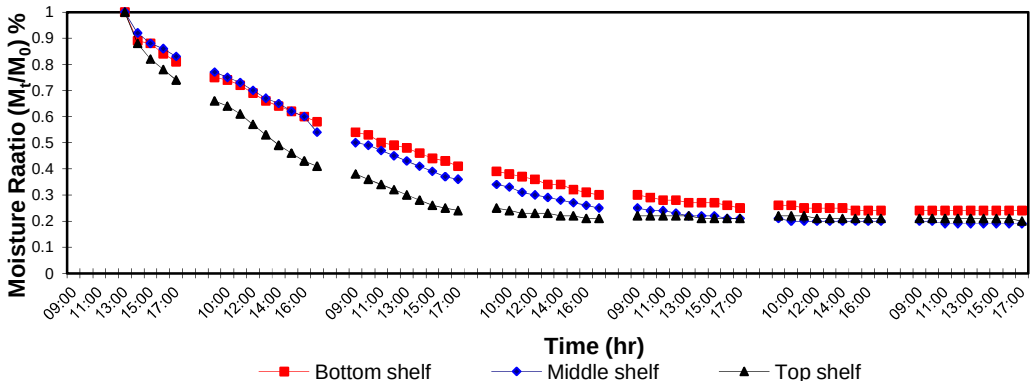

Figure 2: The changes in moisture ratio of unprocessed apricot with time.

The changes in the moisture ratio of sulphated apricot with drying time are shown in Figure 3. The experimental data obtained for air at temperature ranging from 44,8 ^{0}C to 59,7 ^{0}C. There is not any constant-rate drying period in this curve and all the drying operations are seen to occur in the falling rate period. During the falling rate drying period, the drying rate decreases continuously with decreasing moisture content and increasing drying time.

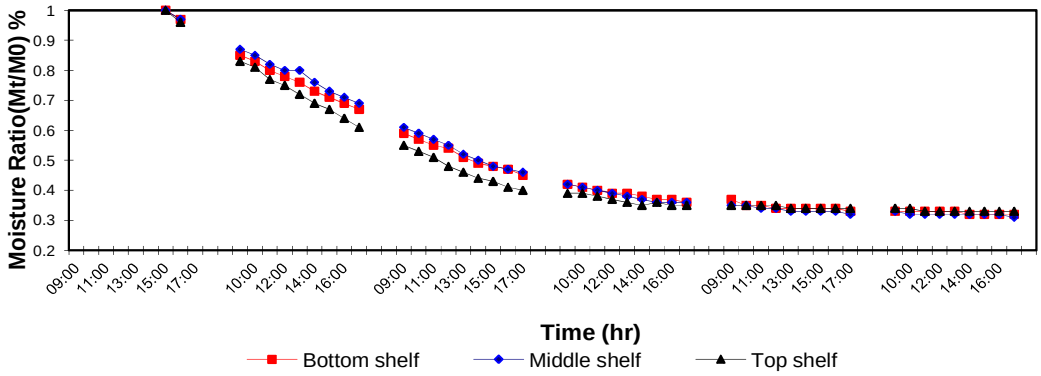

Figure 3: The changes in moisture ratio of sulphated apricot with time.

The effect of these variables on the constant and coefficient of drying expression were also investigated by regression analyses. The drying data as the moisture ratio (MR) versus drying time were fitted to the fourteen drying models. Table 2 for unprocessed apricot drying and Table 3 for sulphated apricot drying show drying model coefficients and the comparison criteria used to evaluate goodness of fit, namely the coefficient of determination (R^2), the reduced Chi-square ($\chi 2$), mean bias error (MBE), the root mean square error (RMSE) and the modelling efficiency (EF) for solar drying. For unprocessed apricot drying the values of R^2, $\chi 2$, MBE, RMSE, and EF for models range from 0,9375 to 0,9982; 0,00010 to 0,00432; -0,01211 to 0,060406; 0,01016 to 0,05989 and 0,8574 to 0,99807 ,respectively. According to Table 2, Midilli and Kucuk model showed good agreement with the experimental data and gave the best result for unprocessed apricot samples.

Table 2. Results of analysis on the modelling of moisture contents and drying time for unprocessed apricot samples.

Model No.	Model name	Coefficients	R^2	RMSE	MBE	χ^2	EF
1	Newton	k=0,03977	0,9652	0,04349	-0,00526	0,0018	0,96529
2	Page	k= 0,05973; n= 0,8754	0,9743	0,03769	-0,00542	0,00128	0,97545
3	Modified Page	k= 0,03999; n=0,8754	0,9743	0,03769	-0,00538	0,00129	0,97545
4	Modified Page Equation	k=0,3603; l= 2,791; n=0,8754	0,9743	0,03803	-0,00545	0,00129	0,97545
5	Henderson and Pabis	a=0,9772; k=0,0387	0,9660	0,04335	-0,00608	0,00174	0,96675
6	Modified Henderson and Pabis	a=0,4636; b=0,7241; c=0,1334; g=0,03289; h=0,03663; k=0,4562	0,9669	0,04436	-0,0015	0,00184	0,96496
7	Logarithmic	a=0,9312; c=0,1486;k=0,06343	0,9950	0,01667	0,060406	0,00432	0,91775
8	Two-Term	a=-15,55; b=16,4; k_1=0,03146; k_2=0,03152	0,9375	0,05989	-0,01211	0,00304	0,94199
9	Two-Term Exponential	a=0,3439;k=0,08328	0,9794	0,03371	-0,00432	0,00104	0,85743
10	Wang and Singh	a=-0,03626;b=0,0003979	0,9901	0,02338	0,00265	0,00050	0,99046
11	Verma et al,	a=0,0037;g=0,04464; k=-0,06103	0,9944	0,01767	-0,00192	0,00024	0,99531
12	Diffusion approximation	a=0,9963;b=-1,363; k=0,04465	0,9944	0,01767	-0,00232	0,00024	0,99531
13	**Midilli and Kucuk**	**a=1,014; b=0,002906; k=0,03306; n=1,157**	**0,9982**	**0,01016**	**0,001056**	**0,00010**	**0,99807**
14	Authors approximation	a=-3,477*10^{-6}; b=0,0006737; c=-0,04118	0,9965	0,01397	-0,00138	0,00014	0,99727

The constants and coefficients of the accepted model for the solar drying of unprocessed apricot drying were as below:

$$(M_t / M_o) = a\exp(-kt^n)+bt, \text{ where } a=1,014;\ b=0,002906;\ k=0,03306;\ n=1,157.$$

Table 3. Results of analysis on the modelling of moisture contents and drying time for sulphated apricot samples.

Model No	Model name	Coefficients	R^2	RMSE	MBE	χ^2	EF
1	Newton	k=0,03389	0,9315	0,05326	0,000240	0,00280	0,93083
2	Page	k= 0,06362; n= 0,8045	0,9593	0,04152	-0,00491	0,00166	0,95895
3	Modified Page	k= 0,03257; n=0,8045	0,9593	0,04152	-0,00489	0,00166	0,95895
4	Modified Page Equation	k=3,784; l= 12,67;n=0,8046	0,9593	0,04199	-0,00485	0,00166	0,95895
5	Henderson and Pabis	a=0,9476; k=0,03139	0,9392	0,05075	-0,00381	0,00248	0,93861
6	Modified Henderson and Pabis	a=0,03134; b=-0,01535; c=1,017; g=4,45; h=0,04852; k=-0,042	0,9936	0,01723	-0,00025	0,00024	0,99349
7	Logarithmic	a=0,8079; c=0,266;k=0,07101	0,9892	0,02162	-0,00018	0,00044	0,98911
8	Two-Term	a=0,03063; b=1,017; k_1=-0,04245; k_2=0,04839	0,9936	0,01683	-0,00032	0,00026	0,99349
9	Two-Term Exponential	a=0,2437;k=0,1019	0,9633	0,03944	-0,00277	0,00150	0,96288
10	Wang and Singh	a=-0,03642; b=0,0004796	0,9913	0,01912	-0,00107	0,00035	0,99119
11	Verma et al,	a=0,008764;g=0,0415;k=-0,06585	0,9904	0,02033	-0,00284	0,00039	0,99030
12	Diffusion approximation	a=0,9912;b=-1,584 k=0,04151	0,9904	0,02033	-0,00297	0,00039	0,99029
13	**Midilli and Kucuk**	**a=1,001; b=0,00598; k=0,02951; n=1,208**	**0,9948**	**0,01518**	**-0,00016**	**0,00021**	**0,99472**
14	Authors approximation	a=-2,112*10^{-6}; b=0,0006133; c=-0,03833	0,9921	0,01841	-0,00248	0,00032	0,99203

For sulphated apricot drying the values of R^2, χ2, MBE, RMSE, and EF for models range from 0,9315 to 0,9948; 0,00021 to 0,00280; -0,00491 to 0,000240; 0,01518 to 0,05326 and 0,93083 to 0,99472, respectively. According to Table 3, Midilli and Kucuk model showed good agreement with the experimental data and gave the best result for sulphated apricot samples. The constants and coefficients of the accepted model for the solar drying of sulphated apricot drying were as below:

$$(M_t / M_o) = a\exp(-kt^n)+bt, \text{ where } a=1,001;\ b=0,00598;\ k=0,02951;\ n=1,208.$$

CONCLUSIONS

In this study, the solar dryer can be used for drying of various agricultural products as well as apricot under the climatic conditions of Izmir. The moisture content was reduced in seven days for unprocessed apricot drying and in six days for sulphated apricot drying. All drying processes occured in the falling rate period. In adddition, the apricot samples of solar dryer were completely protected from birds, insects, rain and dusts.

In order to explain the drying behaviour of unprocessed and sulphated apricots, fourteen different mathematical models were compared according to their coefficient of determination values. According to the results, Midilli and Kucuk model could adequately describe the solar drying behaviour of unprocessed and sulphated apricots in a solar dryer.

It is expected that this system will help growers reduce the cost of drying and obtain more quality dried products. The results of the predicted models showed that used for solar dryers design for different capacity.

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ONE-DIMENSIONAL (FD) MODEL OF TEMPERATURESPREDICTION IN THE CENTER OF PORK MEAT SAMPLE DURING HEAT TREATMENT

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ABSTRACT: The mathematical model can be defined as a set of mathematical relations that describe or define the relationship between certain physical quantities in the reporting process. The aim of this paper was to developed a one-dimensional (FD) model for temperatures prediction during heat treatment of pork meat for non-stationary heat transfer conditions.The pork meat sample was processed in the oven by dry method of heat treatment (one-sided rasting) at three different ambient tremperatures of about 114℃, 152℃ and 204℃ to reach 71˚C in the center of the sample at a given distance x from the bottom surface.The temperature is controlled using a thermocouple.After the heat treatment system, we developed parameters which served to verify the validity of the model. In the end we made an experimental comparison of results with those obtained in the FD model.The developed model showed a high degree of determination and correlation with experimental results, ranging in the interval R = 0,9622 – 0,9948 and determination R^2 = 0,9259 – 0,9897.
Keywords: *FD model, pork meat, heat processing of meat, temperature in the oven*

INTRODUCTION

A model proposed to predict the temperature profiles during one-sided pan-frying of pork meat patties (Zorrillaand Singh, 2003). Enthalpy formulation was used to solve the heat transfer equation due to the phase change in the process.Food and Drug Administration (FDA) recommended that the cooking process of patties to a center temperature should be at least 71℃ or 68,3℃ and h old for 15 seconds (FDA, 1999). Rate of heating to ground pork patties related to some properties such as retention of patty color, texture total crude lipid content, fatty acid, and cholesterol composition (Pan and Singh, 2001). The heat transfer coefficient is an essential parameter in thermal processing of food materials. Beside of the center temperature, soluble protein can be used as an indicator to determine the degree of cooking (Murphyet al., 2002).

Pan and Singh studied ground beef and determined the rate of change in physical and thermal properties of it (Pan et al., 2001). Temperature and holding time affected fat and water holding capacities. In one-sided cooking of pork meat patties, there is a contact between patty and heatingsurface. The rate of heat transfer between them is described by the contact heat transfer coefficient. The contact heat transfer coefficient is a function of heating temperature and time. Physical and thermal properties of heating surfaces, heating temperature and characteristics of medium at the interface may affect the heat transfer rate. Heat transfer process that is predicted using this model is sensitive to fat content. A mathematical model of onesided cooking was developed to predict temperature profiles in meat patties. In this mathematical model the approach of enthalpy formulation was used for heat transfer equation (Ou and Mittal, 2005a).

Transfer processes in meat patties were studied in a one-sided pan fryer. Water and fat losses affect the heat transfer. It was shown that the frying time is a function of fat content and grill temperature. Water loss is increased with the increase in average

temperature (Oroszvári et al., 2005b). Ou and Mittal developed a model of heat and mass transfer for the frying of frozen hamburger patties. Moisture transfer was due to diffusion and capillary flow, but the contribution of diffusion was lower (Ou and Mittal, 2005a). Amézquita et al. studied the effect of various IR-grilling/hot air cooking condition on moisture and fat content and on some physical properties of meat patties. The IR-grilling resulted in crust formation and reduced the fat and moisture content during subsequent convection cooking (Amézquita et al., 2005a).
The aim of the study presented in this paper was to develop 1-D modeling program for temperatures prediction in centre of sample during heat treatment of pork meat for non-stationary heat transfer conditions as function of time and temperature in the oven.Thus, hamburgers were cooked in a one-sided scheme, at a static electrical oven with constant moisture and temperature. A mathematical model of heat transfer was developed to study the change of temperature in the hamburgers. The 1-D modeling program was written in a simulation program (MATLAB 7) along the thickness. The predicted data were compared to experimental ones to validate this modeling process.

MATERIAL AND METHODS

The process of heat treatment

We conducted a dry heat treatment of pork slices samples until the reaching center sample temperature of 71℃ at a given distance "x" from the bottom surface. In doing so, we used three ambient temperatures in the oven "Elit" 3kW, about 114℃, 152℃ and 204℃. Slices were placed between two copper pl ates, a square measuring 10x10 cm. Before the start of heat treatment, copper plates are placed into the oven to reach the temperature of the oven. Slice was placed between the copper plate and heat treated in **the** oven. The process of heat treatment is completed when in the slice center reached a desired temperature. Two K-type thermocouples "Testo" and "HANNA" HI 98 810, (-50˚C and +250˚C) were used to measure surface temperature and the temperature in the center of the sample. The surface temperature of the top copper plate and the temperature inside the oven was measured by analog thermometers. The temperature inside the oven was measured at specific time intervals and expressed as the arithmetic mean value for a given number of measurements. Initial temperature of the samples was around -6℃, to prevent moisture loss. The diameter of the slice was about 90 mm, a thickness of about 14 mm. The initial moisture content was about 60%.
A simple one-dimensional *finite difference* (FD) model was developed, which allows shortening the time required for the calculation of temperature in the center of the sample T_c.This model is based on a numerical method which can predict the temperature in the center of the sample, Tc. Then, carried out to compare results obtained in the FD model with experimental results in order to verify the validity of the model.

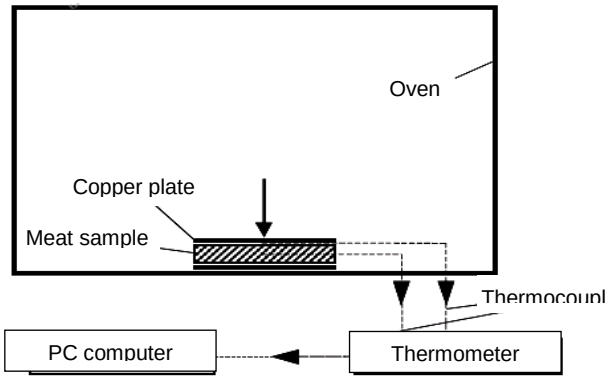

Figure 1. Schematic of experimental conditions and the position of auxiliary components

Table 1. The experimental conditions

Experiment number	Exp.1; Exp.4; Exp.7	Exp.2; Exp.5; Exp.8	Exp.3; Exp.6; Exp.9
Starting sample t℃	-4.54	-4.48	-5.46
The top surface t℃	95	130	189
The bottom surface t℃	112	150	200
The average ambient. t℃	114	152	204
The average time (min)	30	20	15

Table 2. Physical and thermodynamic properties of pork samples

Property	Symbol	Value	Source
Thermal conductivity (W/m℃)	K	0,416	Ou and Mittal (2006)
Heat capacity (J/kg℃)	C_p	3268	Ou and Mittal (2006)
Density (kg/m^3)	ρ	2417	Calculated
Latent heat of fusion (MJ/m^3)	L_f	181,5	Ou and Mittal (2006)
Water conductivity of sample(1/s)	K_w	0,017	Pan and singh (2001)
Coefficient of water holding capacity (1/°C)	δ_{we}	0,0132	Pan and singh (2001)
Coefficient of heat transfer (W/m^2°C)	h	250	Ou and Mittal (2006)
Slice diameter (mm)	D	90	This paper
Slice thickness (mm)	L	14	This paper

Table 3. Distances of nodes considered from the bottom surface of the sample

Nodes	Bottomnode	Δx_1	Δx_2	Δx_3	Δx_4
Distancesfrom the bottom surface (mm)	0	3,5	7,0	10,5	14

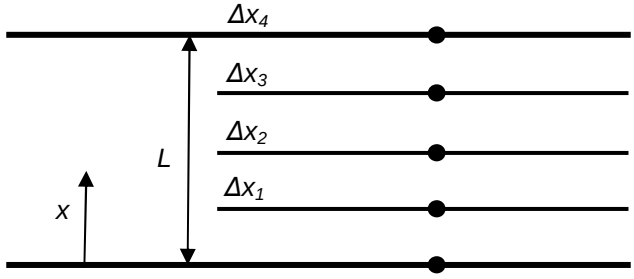

Figure 2. Schematic of nodes considered in the axial direction for finite difference model

Finite difference (FD) model

FD model of heat transfer, presented in this paper, a similar model developed by Ou and Mittal (2006). The process of heat transfer is divided into three phases, the process of heat transfer before the thaw, **in** during thawing and after thawing of the

sample. The assumption is that if the diameter of the slices five times greater than its thickness, in this case, heat transfer can be regarded as one-dimensional in the axial direction. Other important prerequisites for establishing the model of heat transfer involved the assumption of constant physical and thermal properties during heat treatment, constant chemical changes in the sample and the slices were observed as a uniform medium.The assumption is that the top and bottom slice surfaces in contact with the convective transfer coefficientmedium "h_b" (bottom surface) and "h_t" (top surface).

RESULTS AND DISCUSSION

The equations that describe the process of heat transfer in frozen and thawed state for each node can be written as:

Period I (T_0 do 0℃):

Top surface: $K\frac{\left(T_2^j - T_t^j\right)}{\Delta x_4^j} = h_t\left(T_t^j - T_{tp}\right) + \rho C_p \frac{\left(T_t^{j+1} - T_t^j\right)}{\Delta t}$

Node 2: $K\frac{\left(T_c^j - T_2^j\right)}{\Delta x_3^j} = K\frac{\left(T_2^j - T_t^j\right)}{\Delta x_4^j} + \rho C_p \frac{\left(T_2^{j+1} - T_2^j\right)}{\Delta t}$

Node T_c: $K\frac{\left(T_1^j - T_c^j\right)}{\Delta x_2^j} = K\frac{\left(T_c^j - T_2^j\right)}{\Delta x_3^j} + \rho C_p \frac{\left(T_c^{j+1} - T_c^j\right)}{\Delta t}$

Node T_1: $K\frac{\left(T_b^j - T_1^j\right)}{\Delta x_1^j} = K\frac{\left(T_1^j - T_c^j\right)}{\Delta x_2^j} + \rho C_p \frac{\left(T_1^{j+1} - T_1^j\right)}{\Delta t}$

Bottom surface: $h_b\left(T_{bp} - T_b^j\right) = K\frac{\left(T_b^j - T_1^j\right)}{\Delta x_1^j} + \rho C_p \frac{\left(T_b^{j+1} - T_b^j\right)}{\Delta t}$

The initial condition for solving the above equation is: $T(x,0) = T_0$.

Period II (of ice melting):

Top surface: $h_t\left(T_t^j - T_{tp}\right) = L_f \frac{\left(\Delta x_4^{j+1} - \Delta x_4^j\right)}{\Delta t}$

Node T_2: $K\frac{\left(T_2^j - T_t^j\right)}{\Delta x_4^j} = L_f \frac{\left(\Delta x_3^{j+1} - \Delta x_3^j\right)}{\Delta t}$

NodeT_c: $K\frac{\left(T_c^j - T_2^j\right)}{\Delta x_3^j} = L_f \frac{\left(\Delta x_2^{j+1} - \Delta x_2^j\right)}{\Delta t}$

Node T_1: $K\frac{\left(T_1^j - T_c^j\right)}{\Delta x_2^j} = L_f \frac{\left(\Delta x_1^{j+1} - \Delta x_1^j\right)}{\Delta t}$

Period III (0℃ to end of the process):

Equations of this period are similar to the equations of period I.

The amounts of Δt and Δx were selected as followed: $F_0 \le 0{,}5 \Rightarrow \frac{\alpha \Delta t}{(\Delta x)^2} \le 0{,}5$

Thermal diffusivity: $\alpha = \frac{K}{\rho C_p}, \left[\frac{m^2}{s}\right]$

Where the value of Δt and Δx used in this model are 60s and 3,50 mm. Superscript *j*- time numerator.

Combining and solving the previous equation for the nine observed experiments, can be performed equationsof curves, depending on the center temperature "Tc" of the sample with the time "t" required to achieve temperature Tc=f(t). In this way, given the computational data on the value in the center of the sample temperature "Tc", which is then used to compare with as experimental data measured using a thermometer with a thermocouple. In this way, comparing computational and experimental data can be assessed validity of one-dimensional (FD) axial heat transfer model.

Table 4. Equations of curves that describe the dependence of Tc = f (t) for a sample of pork meat processed in oven by dry method (roasting)

Eks. br.	T_c=f(t)	R^2	R	RMSE	V %
	Δx=3,5mm				
Eks.1	T_C =0,0004t^4 - 0,0241t^3 + 0,4881t^2 - 0,2021t - 5,536	0,9644	0,9821	5,3167	15,2634
Eks.2	T_C =-0,0065t^3 + 0,3199t^2 + 0,955t - 6,7999	0,9709	0,9854	5,0511	14,6154
Eks.3	T_C =0,0294t^3 + 0,1034t^2 + 1,7287t - 5,2	0,9578	0,9787	6,4015	17,8068
	Δx=7,0mm				
Eks.4	T_C = 0,0003t^4 - 0,0213t^3 + 0,4941t^2 - 0,9873t - 5,3307	0,9897	0,9949	2,8245	8,1399
Eks.5	T_C = 0,0006t^4 - 0,034t^3 + 0,6708t^2 - 1,3453t - 6,2571	0,9259	0,9623	7,4421	22,1163
Eks.6	T_C = -0,0303t^3 + 0,8084t^2 - 0,5247t - 6,4	0,9559	0,9777	6,2524	19,4626
	Δx=10,5mm				
Eks.7	T_C = 1x10$^{-5}t^4$ - 0,0015t^3 + 0,0562t^2 + 1,1131t - 6,6187	0,9662	0,9830	4,7073	12,6601
Eks.8	T_C = -0,001t^3 + 0,0244t^2 + 2,417t - 7,3177	0,9613	0,9804	5,1769	12,6815
Eks.9	T_C = 0,0006t^4 - 0,0292t^3 + 0,4611t^2 - 0,1402t - 6,2597	0,9667	0,9832	4,7328	14,3917
Prosječno		*0,9621*	*0,9808*	*5,3228*	*15,2375*

Based on the program developed in MATLAB, was performed the correlation of experimental data by the model proposed of Sargolzaei (2009) that created polynomial dependencies, where, as can be seen from the table 4,obtained a high degree of correlation, which ranges in the interval R = 0,9622 - 0,9948, and determination R^2 = 0,9259 - 0,9897.

Standard deviation of regression, or an average deviation of actual (experimental) values of dependent variable (temperature) from regression values, ranging from 2,82 (experiment 4),where the coefficient of variation of 8,14% to 7,44 (experiment 5) with a coefficient of variation of 22,11%.

The following diagram shows the variation of temperature in the oven and the center of the sample compared to the computational results obtained in the FD model and experimental results.

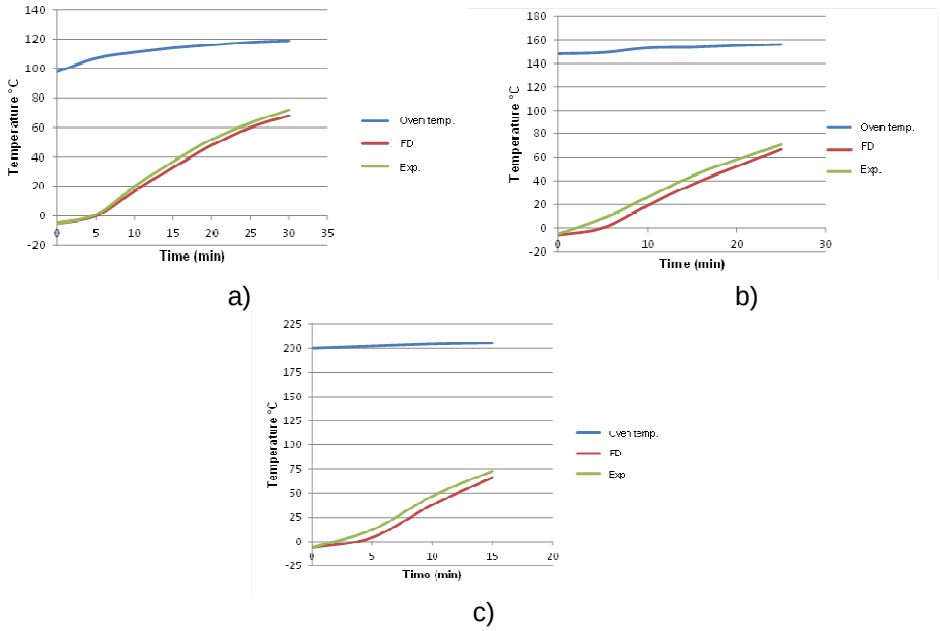

Diagram 1. Comparison of experimental results and the results obtained in the FD model for the three experiments: a)Exp. 2; b)Exp. 5; c)Exp. 8

As shown in the diagram 1 a curve of temperature change in the center of the sample has a very small angle, seen from the beginning to achieve the 0˚C, because the main mechanism of heat transfer during this period is conduction through the solid phase (meat, ice, grease).The amount of heat transferred in this period is not great because of the low coefficient of thermal conductivity of ice. As soon as a thaw of ice, slope will increase as seen from the chart, because the main mechanism of heat transfer **is** fluid (water and fat). This change occurs because water has a much higher coefficient of thermal conductivity than ice.

CONCLUSIONS

A one-dimensional finite difference (FD) mathematical model prediction of temperature in the center of the meat samples at different distances as a function of time and ambient temperature were developed**.** The model showed a high degree of determination and correlation with experimental results, which range in the interval R = 0, 9622 - 0, 9948, and determination R^2 = 0, 9259 - 0, 9897. Standard deviation of regression, or an average deviation of actual (experimental) values of dependent variable (temperature) from regression values, ranging from 2,82 (experiment 4), where the coefficient of variation of 8,14% to 7,44 (experiment 5) with a coefficient of variation of 22,11%.Such a set model developed in Matlab is an important tool in industrial conditions to predict the temperature at the desired distance in the center of the sample with respect to environmental conditions during the heat treatment of the pork meat sample.

ACKNOWLEDGEMENTS

This paper is based on the scientific research project entitled "Effect of heat treatment temperature on protein structure and properties of pork meat." The project is funded by the Ministry of Science and Technology of the Republic of Srpska.

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OPTIMIZATION OF DRYING KINETICS OF BROCCOLI (*Brassica oleracea* L.)

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ABSTRACT: The aim of this paper was to optimize the technological process of broccoli drying. The drying experiment were carried out in the laboratory dehydrator, at two temperature regimes of drying, batch process (constant air temperature of 70 ^{o}C for a period of 8h) and simulated continual process (the air temperature of 70 ^{o}C for a period of 3h and then the air temperature of 50 ^{o}C for a period of 5h). The amount of evaporated water was expressed in relative and absolute units of measure. The experiment was conducted to follow the moisture rate of drying of broccoli and was expressed as speed changes in the ratio of moisture content in the moist material depending on the current moisture content. The results showed that in simulated continual process of drying the lower maximum moisture rate of drying was achieved, drying was more evenly and in this way the technological process was finished with a smaller temperature stress for plant tissue, resulting in a better quality of the final product. The second temperature regime of draying, which simulated continual process, was more acceptable in terms of the final product.

Key words: *broccoli, drying, kinetics, quality*

INTRODUCTION

Broccoli (*Brassica oleracea* L.) is a vegetable with exceptional medicinal and nutritional properties. In Serbian traditional diet broccoli has been poorly presented or not presented at all. Dried broccoli in Serbia has not been produced, but on the international market it has been used in the production of dehydrated soups, sauces even for the tea production. Broccoli is a vegetable with numerous bioactive substances with health-promoting properties. Broccoli is a rich source of glucosinolates and has a high content of flavonoids, vitamins and mineral nutrients. (Moreno et al., 2006). The results of many studies of prevention of certain types of cancer suggest a positive effect on the consumption of vegetables from the cabbage family, especially broccoli (Hecht, 2000; Murillo and Mehta, 2001, Finley et al., 2001; Kristal and Lampe, 2002). Anticancer compound from the group of isothiocyanates, sulforaphane (Jeffery et al., 2003), were determined by the HPLC and ELSD methods, and it was found that the germ contained 10 times larger volume compared to the mature broccoli, and that the concentration of this compound decreased during processing (Nakagawa et al., 2006). In addition, the compound indole-3-carbinol in broccoli has shown anticancer, antioxidant and antiatherogenic properties (Dashwood et al., 1989, Hsu et al., 2006, Higdon et al., 2007).

Preserving by drying is one of the oldest methods of preserving foods in the technological, microbiological and nutritional terms. Drying is a complex process involving heat and mass transfer phenomena. The convective drying method is drying by flow of heated air under controlled and adjusted conditions (Niketić – Aleksić, 1994). Drying of broccoli florets at different temperatures of air and the quality of dried product were studied in the papers of Icier et al. (2010) and Jin et al. (2011).

The aim of this paper was to investigate the two different temperature regimes of broccoli drying in order to show which one has represented the better options for drying. This was achived by determining the rate of the water loss from broccoli florets, not just in a function of time (such analysis can only be indicated to the dryer), but in changing the speed of evaporation of water in relative and absolute units of measure (in % and g) depending on the broccoli current moisture content as has been invasigated in previous papers of apple drying (Paunović et al. 2010; Paunović et al. 2011). The other purpose was to determine which regimes of drying provided the final product with more acceptable characteristics from the technological and organoleptical point of view.

MATERIAL AND METHODS

Broccoli used for experiment was purchased at a local supermarket; the leaves were removed and the florets were separated from stems so that, the diameter of each florets was approximately 1 cm. The broccoli florets were laid out on the three mesh trays of the laboratory dehydrator Stöckli, Switzerland. On the each mesh tray 500 g of the sample was placed. Dehydrator has its own thermostat which controls the heater of 600 watts and maintains a set air temperature. One of the initial assumptions was that in the dryer the same amount of broccoli has always been presented. It was taken care of this, also as the quantity of sample on the mesh trays, for uniform air flow and relation of broccoli air amount. The important initial assumption was that the characteristics of air (T, φ, V) were not changed during the experiment.
The experiment was conducted to follow the drying rate of broccoli florets which was expressed as drying rate changes in the ratio of moisture content of material in the wet material ($\frac{dw}{d\tau}$; %/h) depending on the current moisture content of material (w; %). The kinetics of drying was monitored by two different air temperature regimes as followed:

- sample A – drying at constant air temperature of 70 ^{o}C for a period of 8 h
- sample B – drying at air temperature of 70 ^{o}C for a period of 3 h and then in the next 5 h the air temperature was lowered at 50 ^{o}C

For every 60 minutes the broccoli sample was taken for measurement of moisture content. Each sample was selected by random sampling, in order to reduce experimental error, (from each mesh tray, a certain amount of sample has been taken). The moisture content in each sample was determined by standard gravimetric method.
Moisture content of sample was expressed as the rate of moisture in moist broccoli florets (w; %) and as moisture content in grams which binds 1 g of dry matter of the broccoli (u; g/g). The actual loss of moisture from 100 g of the broccoli florets was calculated Eq. (1):

$$W = G(1 - \frac{C_1}{C_2}) \quad (1)$$

where are: W - mass of evaporated moisture (g),
G - initial mass of fresh broccoli (g)
C_1 - dry matter content in fresh broccoli (%)
C_2 - dry matter content in dried broccoli (%)

RESULTS AND DISCUSSION

The results of determination of the moisture content (w) in the broccoli during drying and the moisture rate of drying expressed as a moisture content over time ($\Delta w/\Delta \tau$) are shown in Table 1.

Table 1. Moisture content in broccoli and moisture rate of drying.

Time (h)	Sample A		Sample B	
	w (%)	$\Delta w/\Delta \tau$ (%/h)	w (%)	$\Delta w/\Delta \tau$ (%/h)
0	84.9	0	85.1	0
1	82.8	2.1	83.1	2.0
2	67.9	14.9	69.2	14.0
3	45.1	22.8	51.9	17.3
4	40.5	4.6	44.1	7.7
5	30.6	9.9	33.5	10.6
6	18.9	11.7	24.0	9.6
7	13.6	5.3	20.7	3.3
8	12.1	1.5	15.1	5.6

w (%) - moisture content; $\Delta w/\Delta \tau$ (%/h) - moisture rate of drying

Based on these results, the graphs on moisture rate of drying depending on the current moisture content of the broccoli florets were constructed and presented in Figures 1 and 2.

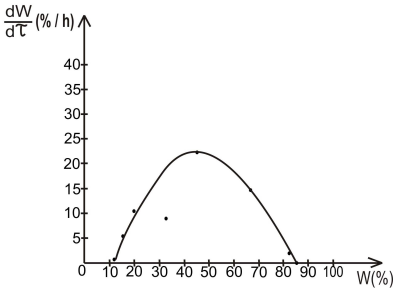

Figure 1. Moisture rate of drying depending of moisture content of broccoli (sample A).

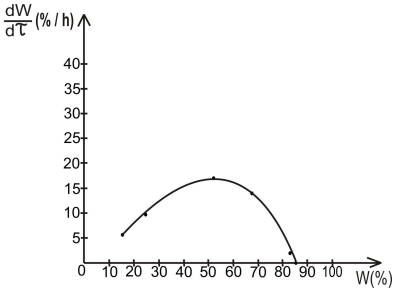

Figure 2. Moisture rate of drying depending of moisture content of broccoli (sample B).

From these figures it can been seen that the moisture rate of drying was gradually increased in both samples and the maximum was achieved at the current moisture content of 45.1 % and 51.9 % for the samples A and B, respectively. After that the moisture rate of drying was gradually decreased. In the sample B the maximum moisture rate of drying was achieved at the lower value compared to the sample A. After that the decreasing of draying rate after reaching the maximum moisture rate of drying was moderate (consistent drying) resulting in a much better quality of the final product. On the other hand, the sample A achieved the maximum moisture rate of

drying at higher value and after reaching it the moisture rate of drying was rapidly decreased, which might cause the stress for the plant tissue, inevitably resulting in worse quality of the final product. These results are in accordance with the previous results of drying kinetics of apple described in the papers of Paunović et al. 2010 and Paunović et al. 2011. It can be concluded that the second regime of draying, which simulated continual process, was more acceptable in terms of the quality of the final product. This indicates that the lower temperatures of air are more favorable for drying the foodstuffs prone to enzymatic browning.

Also, it was significant to determine the actual loss of moisture content from 100 g of the broccoli florets. The total dry matter in fresh broccoli was 15.1 % and 14.9 % for the samples A and B, respectively, while in the dried form it was 87.9 % and 84.9 %. According to the relation Eq. (1), it was calculated that in the sample A from 100 g of broccoli 82.82 g of moisture was evaporated during drying while in the sample B it was 82.45 g.

$$W = 100 \times \left(1 - \frac{15.1}{87.9}\right) = 82.82 \text{ g (sample A)} \qquad \text{(Eq 1)}$$

$$W = 100 \times \left(1 - \frac{14.9}{84.9}\right) = 82.45 \text{ g (sample B)}$$

Since the drying of broccoli lasted 8 hours, this means that the average moisture content loss per hour of drying was approximately 10.35 g of water and 10.30 g of water for samples A and B, respectively. It was also necessary to define the distribution of water according to its activity, ie. the binding energy to the dry matter (Paunović et al., 2010; Paunović et al., 2011).

In order to acchive the percentage distribution of the evaporated amount of moisture content over time, its content was expressed in relation to dry matter. Table 2. shows the data for the calculated values of amount of moisture content in relation to dry matter (u) and evaporated moisture content (in g) during each hour of drying (the starting point was that in the dryer it was 100 g of broccoli).

Table 2. Mass of evaporated moisture content from 100 g of broccoli during drying

Time (h)	Sample A		Sample B	
	Moisture content u (g/g)	Evaporated moisture content W (g)	Moisture content u (g/g)	Evaporated moisture content W (g)
0	5.6225	0	5.7114	0
1	5.4834	12.21	5.5772	11.83
2	4.4967	40.75	4.6443	39.79
3	2.9867	19.53	3.4832	17.40
4	2.6821	2.13	2.9597	4.33
5	2.0265	3.62	2.2483	4.25
6	1.2517	3.14	1.6107	2.79
7	0.9007	1.14	1.3893	0.82
8	0.8013	0.30	1.0134	1.24
Σ	-	82.82	-	82.45

From the Table 2. it can be seen that in the conditions of convective drying the largest amount of moisture content was lost in the first three hours of drying, while during the continuation of the technological process, that amount was significantly reduced. After the first hour of drying 14.74 % and 14.35 % of moisture content was evaporated from the samples A and B, (Fig.3), while after eighth hour of drying 0.43 % and 1.50 % of moisture content from the samples A and B was evaporated, respectively. The amount of evaporated moisture content during 8 h of drying on both air temperature regime is shown in Figure 3.

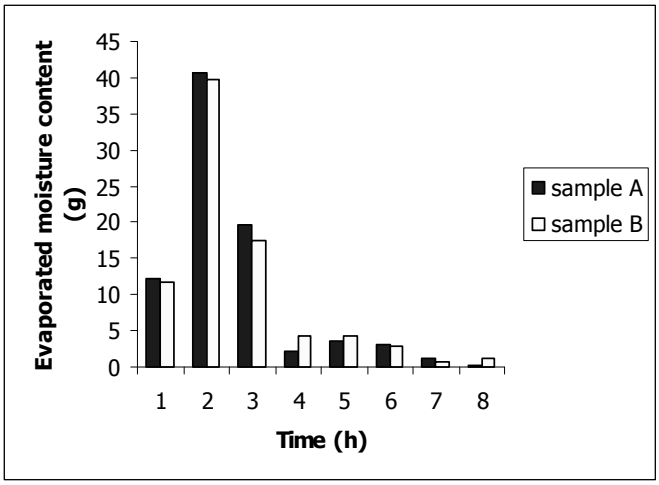

Figure 3. Evaporated moisture content during 8 h drying on both air temperature regime

After the first hour of drying, the broccoli in the both samples started to brown as a result of poliphenol oxidase activity, an enzime that catalyses flavonoid oxidation (Yamaguchi at al., 2003). Browning was intensified during the next two hours of drying. In the fourth and fifth hour in the sample A it was noticed significantly increase of browning, while in the sample B it was slightly reduced than it was observed after the third hour of drying.

The assumption was that the blanching before drying largely prevent oxidation and thus browning of broccoli. However, in the study of Mrkić et al. 2006, it was performed that the blanching and drying processes reduced the ascorbic acid content of broccoli. Also, Yuan et al. (2009) investigated the effect of different ways of processing and the lost of bioactive components in broccoli. They concluded that the content of chlorophyll, vitamin C and glucosinolates decreased after cooking, frying and treatment in a microwave oven, while the smallest lost of these bioactive components was after steam treatment.

CONCLUSIONS

It can be concluded that the second regime of draying, which simulated continual process, was more acceptable in terms of the quality of the final product. The largest amount of moisture content was lost in the first three hours of drying in both air temperature regimes. Following the drying rate of broccoli it can be concluded that the moisture rate of drying increased and reached a maximum at 45.1 % and 51.9 % for samples A and B, respectively. The results showed that in simulated continual process of drying (sample B) the lower maximum moisture rate of drying was achieved, the drying was more evenly and the lower temperature of air was less affected the plant tissue, resulting in a much better quality of the final product. The assumption was that the blanching before drying largely prevent oxidation and thus browning of broccoli. These indications will definitely be re-tested and more precise modeled to accurately optimize the conditions of convective drying of broccoli in order to preserve its bioactive components.

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OPTIMIZATION OF OSMOTIC DEHYDRATION OF APPLE IN SUGAR BEET MOLASSES

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ABSTRACT: Osmotic dehydration is an environmentally acceptable, material gentle drying process, which received considerable attention because of the low processing temperature, base waste material and low energy requirement. Response surface methodology (RSM) was used to determine the optimum processing conditions that yield maximum water loss and dry matter content and minimum solid gain and water activity during osmotic dehydration of apple in sugar beet molasses, and that reduce energy requirements for the process, on the other hand. Temperature (45–65℃), processing time (1–5 h), mol asses concentration (40–80%) were the factors investigated with respect to water loss, solid gain, dry matter content and water activity. Experiments were designed according to 3^3 full factorial experimental designs with these three factors each at three different levels. For each response, second order polynomial models were developed using multiple non-linear regression analysis. Analysis of variance (ANOVA) was performed to check the adequacy and accuracy of the fitted models. The response surfaces analysis showing the interaction of process variables were constructed. Using *Maple 13 Software* optimum operating conditions were found to be at temperature of 45℃, molasses concentration of 80%, and treatment time of 222 min. At this optimum point, water loss, solid gain, dry matter content and water activity were found to be 0.6628 (g/ g initial sample weight), 0.1156 (g/ g initial sample weight), 49.34% and 0.879, respectively.
Key words: *osmotic dehydration, sugar beet molasses, Response surface methodology*

INTRODUCTION

Osmotic dehydration is an effective way to reduce the water content in plant and animal tissue with minimal negative effect on nutritive and sensorial properties of the final product. Osmotic dehydration, used mainly on fruits and vegetables, is performed by immersing them in various hypertonic solutions. Sugar beet molasses can be excellent medium for osmotic dehydration, primarily due to the high dry matter (80%) and specific nutrient content.

The complex cellular structure of plant tissue acts as a, not completely selective, semi-permeable membrane, which allows two main countercurrent flows: water from the plant tissue flows into the osmotic solution whereas osmotic solute diffuses from the solution to the tissue (Kowalska and Lenart, 1998; Rastogi and Raghavarao, 1997; Salvatori et al., 1997). During osmotic dehydration, the tendency is to increase the diffusion of water from the sample into the surrounding solution and decrease penetration of solids from the solution into the plant tissue, on the other hand (Matuska, 2006). However, in the case when sugar beet molasses is used as hypertonic solution, the penetration of mineral substances, vitamins, etc. to the tissue can be considered as favorable because the nutritional value of thus treated fruits and vegetables is higher (Filipčev et al., 2010; Koprivica et al., 2008).

Response surface methodology (RSM) is a statistical approach useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery, 2005).

The first goal for RSM is to find the optimum response. When there is more than one response then it is important to find the compromise optimum that does not optimize only one response (Oehlert, 2000).
The objectives of here presented article were to: 1) investigate the effects of temperature, processing time and concentration on dry matter content (DM), water loss (WL), solid gain (SG) and water activity (a_w); *2)* find the optimum conditions for osmotic dehydration of apple in sugar beet molasses in order to reduce energy requirements and environmental risks, and to reach satisfactory quality of final product, on the other hand.

MATERIAL AND METHODS

Material

Apples (*Idared* type) were purchased from a local market in Novi Sad, Serbia and stored at 4℃ until use. Just before use, apples we re carefully washed, peeled and cut into cubes, dimensions 1x1x1 cm. Initial dry matter content in apple was 14.05±1.49%. The amount of 100g of apple cubes was prepared for each treatment.
Sugar beet molasses was obtained from the sugar factory in Crvenka, Serbia. Initial dry matter content in sugar beet molasses was 80.96%. For the dilution of sugar beet molasses distilled water was used.

Experimental procedure

The process of osmotic dehydration was performed by immersing 100 g of apple sample in a glass jar with the appropriate concentration of osmotic solution in it, which was previously heated to test temperature. All experiments were carried out at atmospheric pressure. To prevent the impact of changes in solution concentration, due to diffusion of water from the sample into solution, 1:10 material/osmotic solution ratio was used. Different concentrations of sugar beet molasses (40.0%, 60.0% and 80.0% dry matter) were used as osmotic solution. The effect of temperature was also investigated and the experiments were conducted at temperatures of 45, 55 and 65ºC. The osmotic dehydration process was performed in a period of 0-5 h under constant conditions. Samples were separated from the osmotic solution at determined intervals of time (1, 3 and 5 h), washed and gently blotted with filter paper in order to remove the excessive water.
Dry matter content of the samples was determined by drying the material at 105 ºC for 24h in a heat chamber (Instrumentaria Sutjeska, Serbia) and measuring the weight loss of the product, gravimetrically on a scale PLJ 360 M**,** KERN with accuracy of ±0.001 g. The solid content of the osmotic solutions was determined refractometrically by Abbe refractometer, Carl Zeis Jenna. Water activity was measured by TESTO 650 (Germany) measurement device with an accuracy of ±0.001 at 25 ºC. All measurements were carried out according to AOAC methods (2000).

Calculations

Evaluation of mass exchange between the solution and the sample during osmotic dehydration were made by using the parameters such as dry matter content *(*DM*)*, water loss (WL*)* and solid gain (SG*)*. In order to account for initial weight differences between the samples, WL and SG were calculated according to the following equations:

$$WL = \frac{m_{s,i} - m_{s,f}}{m_i} \quad \left[\frac{g}{g_{i.s.w}}\right] \tag{1}$$

$$SG=\frac{m_f s_f - m_i s_i}{m_i}\left[\frac{g}{g_{i.s.w.}}\right] \quad (2)$$

where m_i and m_f are the initial and final weight (g) of the samples, respectively; z_i and z_f are the initial and final mass fraction of water (g water/ g sample), respectively; s_i and s_f are the initial and final mass fraction of total solids (g total solids/ g sample), respectively; *i.s.w.* means initial sample weight.

Experimental design and data analysis

In this paper full factorial experimental design was used, with three process variables at three levels (Table 1). The responses measured were WL (Y_1), SG (Y_2), DM (Y_3) and a_w (Y_4).

Table 1. The levels of process variables in coded form

		Coded values		
		-1	0	+1
X_1	Temperature, ^oC	45	55	65
X_2	Time, h	1	3	5
X_3	Concentration	0.4	0.6	0.8

The experimental design, along with values of different responses is given in Table 2.

Table 2. Experimental design and values of experimental data for process optimization

X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4
-1	-1	1	0.4280	0.0663	29.61	0.934	0	1	-1	0.5101	0.0724	34.32	0.926
-1	-1	0	0.3489	0.0442	30.44	0.934	0	0	-1	0.4560	0.0587	32.18	0.93
-1	-1	-1	0.2565	0.0351	21.58	0.931	0	1	1	0.7006	0.1339	50.81	0.871
-1	0	1	0.4562	0.0758	31.76	0.932	0	1	0	0.5976	0.0953	41.60	0.9
-1	0	0	0.3961	0.0526	27.98	0.934	1	1	-1	0.5632	0.0807	38.52	0.917
-1	0	-1	0.3040	0.0359	20.83	0.94	1	-1	1	0.6984	0.1254	53.19	0.858
-1	1	1	0.5092	0.0799	32.95	0.929	1	-1	0	0.5696	0.0891	48.81	0.882
-1	1	0	0.4133	0.0583	28.56	0.934	1	-1	-1	0.4601	0.0639	32.34	0.93
-1	1	-1	0.3409	0.0424	24.49	0.935	1	0	1	0.7276	0.1433	61.12	0.808
0	-1	1	0.6237	0.1060	44.13	0.9	1	0	0	0.6332	0.1064	49.52	0.877
0	-1	0	0.5369	0.0753	43.13	0.904	1	0	-1	0.5090	0.0679	39.65	0.915
0	-1	-1	0.4052	0.0582	28.41	0.934	1	1	1	0.7894	0.1550	64.79	0.783
0	0	1	0.6699	0.1216	47.99	0.883	1	1	0	0.6537	0.1109	50.36	0.871
0	0	0	0.5708	0.0882	40.18	0.914							

A model was fitted to the response surface generated by the experiment. The model used function of the variables:

$$Y_k=f_k(\text{temp., time, conc.}) \quad (3)$$

The following second order polynomial (SOP) model was fitted to the experimental data. Four response equations were obtained depending on three examined variables:

$$Y_k=\beta_{k0}+\sum_{i=1}^{4}\beta_{kI}X_i+\sum_{i=1}^{4}\beta_{kII}X_i^2+\sum_{i=1}^{3}\sum_{j=1+1}^{4}\beta_{kIJ}X_iX_j \quad (4)$$

where β_{kij} are constant regression coefficients; *Y,* either *WL* (Y_1), *SG* (Y_2), *DM* (Y_3) and a_w (Y_4); X_1, osmotic temperature; X_2 treatment time and X_3, solution concentration.
Analysis of variance (ANOVA) and response surface regression method (RSM) were performed using *StatSoft Statistica* 10. The model was obtained for each dependent variable (or response) where factors were rejected when their significance level was less than 90%. Optimal combination of the tested variables was found using *Maple 13 Softwar*e.

RESULTS AND DISCUSSION

Four models were obtained as a result of fitting Eq. 4 to experimental data shown in Table 2. These models were tested for adequacy and fitness by analysis of variance (Table 3). Results showed that developed models for all four responses were significant (high value of r^2) with no significant lack of fit indicating that they adequately described relationship between responses and independent variables.

Table 3. Analysis of variance (ANOVA) for four responses

Source	Par.		Sum of squares and t-test							
		df	WL	t(WL)	SG	t(SG)	DM	t(DM)	a_w	t(a_w)
Linear	t	1	0.26*	51.07	0.01*	37.29	2007.81*	21.36	0.02*	-15.21
	T	1	0.03*	17.82	0.00*	13.63	67.15*	3.91	0.00*	-3.82
	C	1	0.18*	42.70	0.01*	40.60	1152.11*	16.18	0.01*	-12.45
Quad.	t^2	1	0.02*	-14.86	0.00*	-8.86	28.18*	-2.53	0.00^{ns}	-1.25
	T^2	1	0.00^{ns}	-0.55	0.00^{ns}	-0.40	0.35^{ns}	-0.28	0.00^{ns}	0.11
	C^2	1	0.00^{ns}	-0.43	0.00*	3.90	19.57**	-2.11	0.00^{ns}	-0.69
Cross product	t·T	1	0.00^{ns}	1.41	0.00*	3.37	18.61**	2.06	0.00*	-3.25
	t·C	1	0.00*	5.54	0.00*	10.39	141.23*	5.67	0.01*	-10.01
	T·C	1	0.00^{ns}	-1.27	0.00*	3.32	3.64^{ns}	0.91	0.00*	-3.05
Error	Lack of fit	17	0.00^{ns}		0.00^{ns}		74.783^{ns}		0.00^{ns}	
	r^2		0.9966		0.9951		0.9787		0.9685	

*Significant at 95% confidence level, **Significant at 90% confidence level, ^{ns} Not significant, df – degrees of freedom

ANOVA test showed that all linear terms significantly contribute to the formation of statistically significant SOP models. All linear members are significant at 95% confidence level for all four responses. Results indicated that immersion time was the most important factor for WL, DM and a_w, then solution concentration and temperature at the end. Greatest influence on the SG has the solution concentration, then the immersion time and temperature, respectively.
Regression coefficients of the model (β's) are given in Table 4, as well as theirs statistical significance. Knowing those parameters allowed prediction of response values, if the independent variables are known.
Response surface plots (not given in the paper) show that WL and SG rapidly increase at beginning of the process, primarily due to big difference in osmotic pressure between sugar beet molasses and apple samples. After 2.5 - 3 hours WL approaches equilibrium value and intensity of water diffusion decrease, while SG values still rising and solids penetrate in the sample. Increasing temperature, concentration and immersion time caused higher WL, SG and DM, but lower a_w. The temperature dependence of DM and a_w is stronger at the later stages compared to the beginning of the process, but this increasing was the result of penetration of solute into the apple, not the result of water loss (drying). Relationship between SG and a_w was explained in the study of *Eren* and *Kaymak-Ertekin (2007).*

Table 4. Values of the SOP regression coefficients for the four responses

	Y_1	$t(Y_1)$	Y_2	$t(Y_2)$	Y_3	$t(Y_3)$	Y_4	$t(Y_4)$
β_0	-0.32*	-2,31	0.03 ns	0,69	-2.48ns	-0,09	0.75*	6,22
β_1	0.12*	10,56	0.01*	2,40	-0.04ns	-0,02	0.05*	5,36
β_{11}	-0.02*	-14,86	-0.00*	-8,86	0.54*	-2,53	-0.00ns	-1,25
β_2	0.01ns	1,55	0.00 ns	0,15	0.11ns	0,11	0.00ns	0,58
β_{22}	-0.00ns	-0,55	-0.00 ns	-0,40	-0.00ns	-0,28	0.00ns	0,11
β_3	0.53*	3,63	-0.14*	-3,29	53.31**	1,72	0.35*	2,68
β_{33}	-0.04ns	-0,43	0.11*	3,90	-45.15**	-2,11	-0.06 ns	-0,69
β_{12}	0.00 ns	1,41	0.00*	3,38	-0.06**	2,06	-0.00*	-3,25
β_{13}	0.04*	5,54	0.02*	10,39	-8.58*	5,67	-0.06*	-10,01
β_{23}	-0.00ns	-1,27	0.00*	3,32	-0.28ns	0,91	-0.00*	-3,05
R^2	0.9966		0.9951		0.9787		0.9685	

*Significant at 95% confidence level, **Significant at 90% confidence level, ns Not significant

A graphical multi-response optimization technique was used to determine optimum conditions for osmotic dehydration of apple in sugar beet molasses. Optimum conditions for osmotic dehydration of apple cubes were determined in order to reduce energy requirements (lower immersion time and processing temperature) and, at the same time, to get product with satisfactory quality characteristics (high DM and WL, and low a_w and SG). Sugar beet molasses is by product of sugar producing and it has high dry matter content, therefore, in optimization, 80% sugar beet molasses is selected as solution concentration. Range of temperatures and immersion times were determined to reach following values of responses: WL 0.6 - 0.7 g/$g_{i.s.w.}$, SG 0.1 - 0.12 g/$g_{i.s.w.}$, a_w 0.87 - 0.88 and DM 45 - 55%. Requested values for SG are higher than stated in study of *Eren* and *Kaymak-Ertekin (2007),* because for lower SG values, satisfactory low a_w value cannot be achieved.

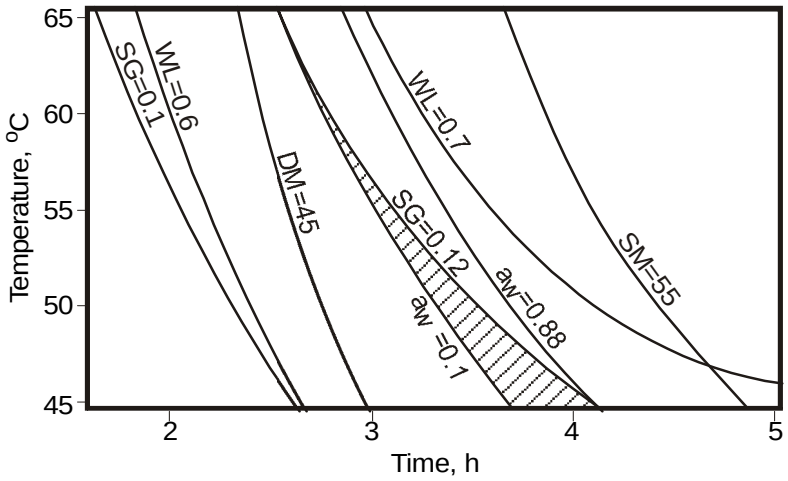

Figure 1. The range of temperature and time (marked) for osmotic dehydration of apples in 80% sugar beet molasses where WL ranges from 0.6 to 0.7 g / $g_{i.s.w.}$, SG from 0.1 to 0.12 g /$g_{i.s.w.}$, a_w from 0.87 to 0.88 and SM from 45 to 55%

Marked area in Figure 1 shows the range of time and temperature in which above values of responses can be achieved. The values of temperature and time range from 45 - 60 ºC and 2.8 – 4.05h, respectively. With increasing temperature setpointed response values were achieved in a shorter time of dehydration and vice versa.

Dehydration at higher temperatures has a negative effect on the nutritional characteristics of the product and increases the cost of the process. In the Table 5 two combinations of parameters that achieve the requested outputs are given. Recommended conditions are to perform dehydration at lower temperatures and shorter time. Response values in the table were obtained from a SOP which are defined for four responses of the system.

Table 5. Optimal conditions for osmotic dehydration of apples in 80% sugar beet molasses

Temperature, ºC	Time, h	Responses
60	2.8	WL=0.6687; SG=0.1202 a_w=0.879; SM=47.57
45	3.7	WL=0.6628; SG=0.1156 a_w=0.879; SM=49.34

CONCLUSIONS

According to applied RSM analysis, all responses (WL, SG, DM and a_w) were significantly influenced by investigated process parameters (immersion time, process temperature and solution concentration), during the osmotic dehydration of apple cubes in sugar beet molasses. Developed SOP models showed good correspondence to experimental data, at statistically significant level $p<0.05$, with the coefficient of determination $r^2>0.96$, for all cases. Determination of optimal conditions was accomplished by applying SOP regression coefficients to nonlinear analysis within Maple 15 code. The maximum WL and DM and minimum SG and a_w values correspond to temperature 45℃, molasses concentration of 80% and immersion time of 3.7h.

ACKNOWLEDGEMENTS

The authors acknowledge financial support of the Ministry of Education and Science of the Republic of Serbia (Project: III - 46005, 2011-2014).

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OPTIMIZATION OF THE SPECIALTY BREAD FORMULATION CONTAINING SUGAR BEET MOLASSES, FLAX SEED AND VITAL WHEAT GLUTEN

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ABSTRACT: In order to improve the specific volume and textural properties of specialty bread made with molasses, the following natural ingredients were added: vital wheat gluten and flax seed. Individual and interactive effects of the added ingredients on specific volume, hardness, springiness and cohesiveness were evaluated using the method of response surface methodology (RSM). Effects were mainly related to the doses of the tested ingredients. In general, the increased doses of vital gluten increased bread volume and flax seed improved bread crumb resilience. Since there was a strong dose dependent relation among the tested attributes, the optimal doses of the ingredients were determined through the desirability function. The aim of the optimization was to maximize the specific volume and minimize hardness. It was found that the optimized doses for ingredients were the following: 5% of sugar beet molasses, 8% of flax seed and 3.96% of vital gluten. The estimated values for specific volume were 4.55 ml/g and hardness 953.25 gf.
***Key words**: sugar beet molasses, vital wheat gluten, flax seed, textural properties of bread, optimization*

INTRODUCTION

Sugar-beet molasses is a thick concentrated liquid syrup, by-product of the processing of sugar beet into sugar. In general, beet molasses contains approximately 50% of sucrose, 1% of farinose, and 0.25% of glucose and fructose by dry weight. The non-sugar compounds present in molasses include many important micronutrients such as minerals (potassium, calcium, sodium, magnesium and iron) and vitamins (B vitamins) (Šušić and Sinobad, 1989; Hickenbottom, 1996). Since sugar beet molasses alone has not been convenient for consumption for most people because of its palatability, the general idea of this research work was to include beet molasses in staple food such as white wheat bread to improve its nutritive value and functionality (Ranhorta, 1998; Lević et. al. 2005; Pribiš et al. 2008).
Previous research work showed that molasses decreases bread volume and increases crumb hardness, especially at higher doses (15% of flour basis) (Lević et al. 2005; 2006). In order to ameliorate these undesirable effects of molasses, natural dough and bread improvers were applied: vital wheat gluten and flax seed.
Vital wheat gluten, a by-product of wheat starch extraction process, is characterized by high protein content (75-85% of dry basis) and good viscoelastic properties (Stenvert et al., 1981). The addition of vital gluten increases dough stability, improves gas retention (Hoseney, 1984), loaf volume, crumb grain, texture, softness and shelf life (Stenvert et al. 1981; Wiepert and Zwingelberg, 1992).
Nowdays, flax seed is increasingly applied in human nutrition and production of functional food and bakery goods due to its nutritive and health value (Payne, 2000). Besides, flax seed is important from technological point of view due to a large quantity of pentosans (7-9%) and dietary fibers (24.5%), both soluble and insoluble

ones (Lagrange, 1995). It is known that pentosans have great capacity of water absorption. Due to that reason they influence the improvement of rheological dough capacities, the increase of volume and preserving of bakery goods freshness (4.7). By the addition of flax seed (8-16% of basis into flour) it can be achieved an increase in bread volume, improvement of crumb quality, aroma, taste and crumb and crust mastication of bakery goods (Bojat et al. 2000).
The main objectives of this study, therefore, were firstly to establish the factors (sugar-beet molasses, vital gluten and flex seed) affecting bread quality and secondly to determine optimum levels of these ingredients for maximum bread quality, especially to improve the specific volume and reduce crumb hardness.

MATERIALS AND METHODS

Commercially refined wheat flour with the following characteristics was used: 13.1 g/100 g water content, 0.52 g/100 g d.b. (dry basis) ash content and 11.3 g/100 g d.b. protein content. Moisture, crude protein, crude ash of flour were determined according to the standard ICC procedures (ICC, 2001).
The characteristics of the applied sugar beet molasses were the following: sucrose content 52.32%, water content 14.96%, ash content 17.91% d.b., NaCl 0.01% and mineral content K, Na, Mg, Fe, Ca is: 21102 mg/kg, 1178.40 mg/kg, 260.92 mg/kg, 102.12 mg/kg, 2110.17 mg/kg, respectively (ICC, 2001).
Chemical analyses of sugar beet molasses were performed according to AOAC methods (AACC, 2003). Mineral content of molasses was determined by atomic absorption spectrometry with an atomic spectrometer “Varian”, model “Spectra 10”.
Vital gluten was applied with the following characteristics: moisture content 8.06%, crude protein content (Nx6.25) 82.17%, crude fat content 2.21% and ash content 0.87%.
Reddish-brown flax seed of domestic origin was used with the following chemical composition: protein content 26.7% d.m., oil content 42.5%d.m., pentosans content 8.0% d.m. Flax kernels before the dough mixing were prepared by soaking with the addition of two times more water quantity at the temperature of 40°C during minimally an hour.
Baking tests were performed under laboratory conditions using rapid mixing procedure. The ingredients used (calculated on flour basis) for the basic wheat dough were the following: flour (100%), fresh yeast (5%), sugar (1%), salt (2%), fat (1%) and water according to farinograph water absorption (FWA) (ICC standard No. 115) (ICC, 2001). Sugar beet molasses (SBM), vital wheat gluten (VWG) and flax seed (FS) were added according to the experimental plan shown in Table 2. Dough preparation and baking were done according to the method described in detail in the work of Filipčev et al. (2010).

Evaluation of bread quality

Quality analysis of fresh bread samples was carried out by measuring weight, volume (determined by seed displacement in a loaf volume meter) and specific volume. Specific volume was calculated as the ratio between volume and mass. Bread texture attributes were evaluated 24h after baking application ‘‘Texture Profile Analysis’’ (TPA). Slices of 2 cm thickness were compressed to 60% of their original height in a double compression test (TPA), at 0.5 mm/s speed test, with a 75 s delay between the first and second compression. Parameters (hardness (gram-force, gf), springiness and cohesiveness) were collected from the TPA graphic.

Experimental design and statistical methods

An incomplete 3^3 factorial design and three replicates at the central point were used to investigate the effect of the three independent variables of physical and sensory properties of the speciality bread (Table 1) (Box & Benhken, 1960).

Table 1. Real and coded values of independent variables used for experimental design

	Independent variables	Coded values		
		-1	0	+1
Real values	Sugar beet molasses, %	5	10	15
	Flax seed, %	4	6	8
	Vital wheat gluten, %	0	2	4

The independent variables SBM, FS and VWG) were assessed at three equidistant levels of variation. For each response (specific volume (SV), hardness (HD), springiness (SP) and cohesiveness (COH)) a quadratic model was used:

$Y= b_0+ b_1X_1 +b_2X_2 +b_3X_3 +b_{11}X_1^2 +b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 +b_{23}X_2X_3,$

Where Y is the response function of the experimental data, X_1, X_2 and X_3 are the independent variables, and b parameters are the estimated coefficients. The adequacy of the model was evaluated by the coefficient of determination, R^2, F-value and model p-value at the 0.1 significance.

The significance of regression coefficients was assessed by p-values at 0.1 significance level. For the optimization of the responses we applied the Design-Expert 8.1 (Stat-Ease Corporation, Minneapolis, MN, USA)_using the desirability function. The desirability function approach (DFA) was used to simultaneously optimize both specific volume and hardness of the specialty bread. Table 4 shows the conditions of the optimization process.

RESULTS AND DISCUSSION

Fifteen experiments were performed according to the experimental design and the results are shown for SV, HRD, SP and COH in Table 2.

Table 2- Experimental design and obtained results

Run	SBM (%)	FS (%)	VWG (%)	SV (ml/g)	HRD (gf)	SP	COH
1	-1	0	-1	4.2	1361.16	0.91	0.54
2	-1	0	1	4.4	953.27	0.92	0.53
3	1	0	-1	2.7	3990.16	0.65	0.35
4	1	0	1	3.1	2798.61	0.75	0.37
5	0	-1	-1	3.9	1346.77	0.90	0.47
6	0	-1	1	3.9	1488.46	0.89	0.49
7	0	1	-1	3.8	2234.09	0.81	0.46
8	0	1	1	3.8	1761.48	0.87	0.46
9	-1	-1	0	4.2	1274.98	0.90	0.51
10	1	-1	0	3.2	2596.09	0.76	0.39
11	-1	1	0	4.6	1323.82	0.86	0.52
12	1	1	0	3.0	3369.62	0.73	0.37
13	0	0	0	3.8	1891.58	0.87	0.46
14	0	0	0	3.8	1995.43	0.83	0.47
15	0	0	0	3.8	2135.71	0.86	0.45

For each response group a quadratic equation was formed with relevant terms ($p<0.1$) to obtain as high R^2 values as possible. The obtained regression equations, R^2 values, F-values and model p-values are presented in Table 3.

Table 3. Effects of factors expressed as their corresponding coefficients obtained in the models for specific volume (SV), hardness (HRD), springiness (SP) and cohesiveness (COH) of the specialty bread formulation containing sugar beet molasses, flax seed and vital wheat gluten[a]

[b]Texture properties	[c]Regression equations	Coefficient of determination (R^2)	[d]F-test	Model p-value
SV	4,525 + 0,065SBM + 0,005SBM*VWG - 0,012SBM^2 + 0,006VWG^2	0.983	31.17	0.001
HRD	-855,221+388,223VWG -154,149SBM-671,349FS +14,033SBM^2	0.961	13.56	0.005
SP	1,043+0,017SBM -0,041FS-0,002SBM^2 +0,017FS^2	0.945	9.60	0.011
COH	0,596 +0,002SBM -0,001SBM^2	0.979	25.71	0.001

[a]Only values of significant coefficients are presented (90% confidence level)
[b]SV-specific volume; HD-hardness; SP-springiness; COH-cohesiveness
[c]SBM-the concentration of sugar beet molasses; VWG- the concentration of vital weat gluten
[d]F-test>F_{listed} (4.735)

On the basis of the results in Table 3, all the models had a coefficient of determination (R^2) higher than
0.94, showing that more than 94% of the total variations around the mean values were provoked by the experimental conditions. Molasses significantly affects ($p<0.1\%$) bread specific volume, and by quadratic increase of its dose specific volume significantly decreases. However, increasing gluten concentration significantly increases bread volume which led to positive interaction effect between molasses and wheat gluten (+0,005SBM*VWG). The addition of flax seed did not considerably affect bread volume.
All the three ingredients at their initial doses significantly affect crumb hardness. At the tested dose interval, vital wheat gluten significantly increases crumb hardness but the quadratic effect of vital gluten dose reduces crumb hardness (although not significantly). Molasses is an important ingredient that affects bread crumb structure: at low doses, molasses softens bread crumb, whereas with quadratic dose increase, it hardens the crumb. This is probably due to its composition, i.e. high sucrose content of 52.32%. It is known that sucrose at 5% dose positively affects the textural properties of bread crumb, whereas higher doses generally produce negative effect on bread quality (Colliery, 1967; Payer, 1973). Flax seed significantly decreases crumb hardness at the tested doses (4-8%), whereas quadratic effect does not have any further significant effect.
Bread crumb springiness is significantly affected by doses of molasses and flax seed. At lower doses, molasses improves crumb springiness, whereas with quadratic dose increase molasses decreases crumb springiness. Flax seed produces effects on bread crumb opposite to those of molasses. Positive effect of flax seed on crumb springiness is due to its high content of soluble and insoluble dietary fibers and pentosans (Lagrange, 1995; Payne, 2000).
Bread crumb cohesiveness was affected only by molasses and its action depends on the dose. At lower applied doses (5%-10%), the intensity of internal bonds increases in bread crumb, whereas crumb stability decreases with quadratic increase in molasses dose. The addition of molasses imputs into bread which lowers crumb cohesiveness. This is in accordance with the findings of Collyer (1967), who concluded that higher sucrose doses affect the consistency of baked products by reducing crumb cohesiveness.

Process optimisation through the desirability function

In Table 4, the conditions of the optimization process to maximize specific volume and minimize hardness in the specialty bread are displayed. The selection of bread

quality attributes (responses) in the optimization process and their relative importance was based on literature data and consumer preference (Pestorić et al. 2011). The simultaneous evaluation of the experimental design responses which resulted in the overall desirability profile are shown in Fig.2. By applying the desirability function, the optimum concentrations were sugar beet 5% of molasses, 8% of flax seed and 3.96% of vital wheat gluten into flour, with a desirability value of 0.984. In these concentrations, maximum specific volume and minimum hardness were obtained, showing predicted values of 4.55 ml/g and 953.255 gf, respectively.

Table 4. Conditions and obtained results of the optimization process to maximize specific volume and and minimize hardness of the specialty bread with sugar beet molasses, flax seed and vital wheat glute

Factors and responses	Goal	Lower limit	Upper limit	Importance	Optimum
Vital wheat gluten (%)	in range	0	4	3	3.96
Sugar beet molasses (%)	in range	5	15	3	5.00
Flax seed (%)	in range	4	8	3	8.00
Spec Volume (ml/g)	MAX	2.7	4.6	5	4.55
Hardness (gf)	MIN	953.266	3990.16	3	953.25

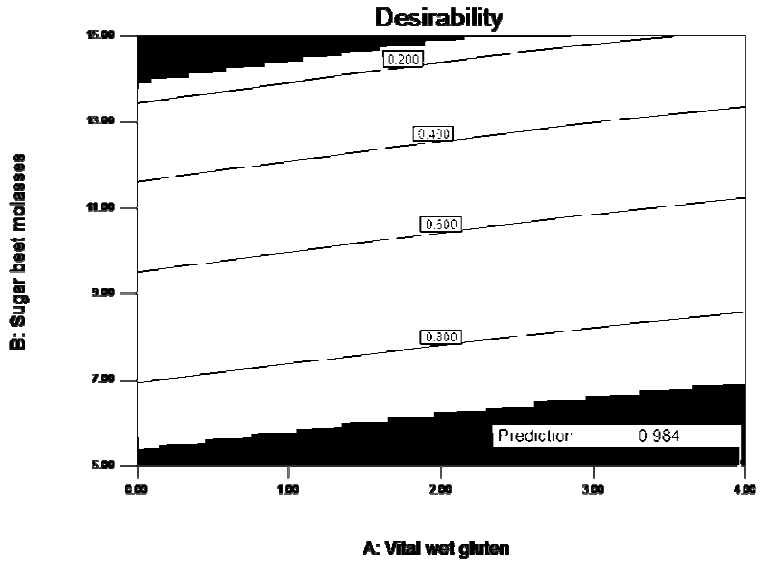

Figure 2. Dependence of overall desirability function on the dose of sugar beet molasses and vital wheat gluten in the specialty bread formulation containing sugar beet molasses, flax seed and vital wheat gluten factors (flax seed) was set to its constant (medium) value

CONCLUSIONS

Upon the analysis of the presented results, the application of natural ingredients like flax seed and vital wheat gluten to improve the quality of the specialty bread enriched with molasses seems to be justified. Vital wheat gluten produced significant positive effect upon bread specific volume and flax seed improved crumb elasticity and lowered bread crumb hardness. The intensity of these effects was dose dependent. In this paper, we successfully applied the statistical method for the optimization of concentration of natural raw materials like gluten and flax seed aiming at achieving maximal bread quality. The final result of the optimization suggested that the optimal ingredients doses to achieve bread with maximal volume and minimal crumb hardness amounted to 3.96% for vital gluten, 8% for flax seed and 5% for molasses. The estimated values of specific volume and crumb hardness in optimally formulated bread were 4.55 ml/g for specific volume and 953.25 gf for hardness. Beside the

achieved improvement of bread quality, the added flax seed also contributes to the improvement of bread nutritive value, which is the topic of some researches to come.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 031055 financed by the Ministry of Education and Science, Republic of Serbia.

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OSMOTIC DEHYDRATION OF PORK IN THREE DIFFERENT SOLUTIONS-MASS TRANSFER KINETICS

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ABSTRACT: The presented paper describes an investigation of osmotic dehydration of pork (*M. triceps brachii*) in three different osmotic solutions (sugar beet molasses, ternary solution and combination of these solutions in a 1:1 ratio) under atmospheric pressure and room temperature (20^{o}C). The main objective was to examine the influence of different osmotic agents and immersion time on the mass transfer kinetics during osmotic treatment. The most important kinetic parameters of the process: water loss (WL), solid gain (SG), water loss/solid gain ratio (WL/SG), weight reduction (WR), normalized solid content (NSC) and normalized moisture content (NMC) were determined after 1, 3 and 5 hours of dehydration. Mass transfer coefficients were calculated using Hawkes and Flink's model. The results indicate that all three solutions are satisfying osmotic agents and that extensive dehydration occurred during the first 3 hours of the process.
Key words: *Osmotic dehydration, pork meat, mass transfer kinetic, sugar beet molasses, ternary osmotic solution*

INTRODUCTION

In recent years, the process of osmotic dehydration (OD) is considered an important tool for preserving fruits and other raw materials. Osmotic dehydration is used as a pretreatment for many processes, to improve nutritional, sensorial and functional properties of food without changing its integrity. This technique also is interesting because it provides partial water removal from a food product, with low energy consumption and mild heat treatment (*Vieira* at al., 2012; Manivann at al., 2011). At the Faculty of Technology in Novi Sad, a method has been developed for osmotic drying in sugar beet molasses as hypertonic solution. Sugar beet molasses appears to be an excellent medium for osmotic dehydration, primarily due to the high content of dry matter (80%), which provides high osmotic pressure in the solution as well as the specific chemical composition, characterized by high contents of vitamins, minerals, antioxidants and betain (Šušić at al., 1989; Kowalska at al., 1998; Mišljenović at al., 2009).

Physicochemical, sensory and technological properties of fresh meat are related with water content. Water is held in myofibrils, functional proteins of meat, but also it may exist in the intracellular space between myofibrils and sarcoplasm. The water content in meat depends on many factors, including the tissue itself and how the product is handled (time, temperature, treatments) (Barat at al., 2009) .The knowledge of the kinetics of water and salt transfers during the processing is of great technological importance because it allows estimating the immersion time of meat cuts in an osmotic solution to obtain products with determined salt and moisture contents (Schmidt at al., 2009).

The difference in the chemical potential of water between the food and the osmotic medium is the driving force for dehydration. In this process, two counter-current mass transfer flows take place in cell wall of the food: water loss from the food to the solution and solute gain from the solution to the food. The existence of these

simultaneous and opposite fluxes is one of the major difficulties in modeling osmotic dehydration kinetics (Koprivca at al., 2010; Mercali at al., 2010). Temperature, osmotic solution concentration and total processing time are the most important variables in osmotic process. Increasing the osmotic solution concentration induces an increase in the mass transfer (Ferrari at al., 2011; Corrêa at al., 2010; Silva at al., 2012). Great influence on the kinetics of water removal and solid gain has the type of osmotic agent. Ternary aqueous solutions containing salt and sugar are usually used as osmotic agents for meat dehydration (Damez at al., 2008; El-Aouar at al., 2006).
This study was aimed at investigating the influence of different osmotic solutions and immersion time at room temperature on the efficiency of osmotic dehydration process of pork. Kinetics parameters, mass transfer coefficients for water, and rate of mass transfer were defined.

MATERIAL AND METHODS

Pork (*M. triceps brachii*) was purchased at the local butcher shop in Novi Sad, shortly before use. Initial moisture content of the fresh meat was 72.83%. Prior to the osmotic treatment, fresh meat was cut into cubes, dimension of nearly 1x1x1cm. As hypertonic medium three different solutions were used.The first one, ternary osmotic solution, was made from sucrose in the quantity of 1.200 g/kg water, NaCl in the quantity of 350 g/kg water and distilled water (in further text indicated as solution 1). The second osmotic solution was combination of the first and third in ratio 1:1 (in further text indicated as solution 2). The third, sugar beet molasses, with inital dry matter content of 85.04%, was obtained from the sugar factory Pećinci, Serbia (in further text indicated as solution 3). The material to solution ratio was 1:5 (w/w). Dehydration was performed at room temperature (20^{o}C), with stirring on every 15 minutes under atmospheric pressure. Samples from all three solutions after 1, 3 and 5 were taken out to be lightly washed and gently blotted, to remove excess water. Dry matter content of the fresh and treated samples was determined by drynig at 105^{o}C for 24h in a heat chamber (Instrumentaria Sutjeska, Serbia) until constant weight. All analytical measurements were carried out in accordance to AOAC (2000).
In order to follow mass transfer kinetics of the OD, three key process variables were measured: moisture content, change in weight and change in the soluble solids. Using these, water loss (WL), weight reduction (WR), solid gain (SG), normalized moisture content (NMC), normalized solid content (NSC) were calculated, also mass transfer coefficients were obtained using Hawakes and Flink's model as described by Mišljenović at al., 2009.

RESULTS AND DISCUSSION

Table 1 shows changes in dry matter content in the samples of pork meat during OD as a function of different type of osmotic solution and dehydration time. The increase of immersion time during the process resulted in higher dry matter content in pork samples and the highest value was achieved in solution 3 after five hours of immersion (60.07%).
Along with changes in dry matter content, changes in kinetic parameters occurred and they are as well shown in table 1.

Table 1. Changes of dry matter content and the kinetic parameters during osmotic dehydration of pork meat

Type of osmotic solution	Time (h)	Dry matter, (%)	WR, g/g initial sample weight	SG, g/g initial sample weight	WL, g/g initial sample weight	WL/SG
Solution 1	1	42.96	0.17032	0.093922	0.243504	2.592619
	3	51.75	0.250872	0.128663	0.355132	2.760172
	5	54.90	0.291313	0.122204	0.404643	3.311209
Solution 2	1	41.17	0.179362	0.073694	0.234615	3.183638
	3	49.83	0.303366	0.089973	0.364276	4.048726
	5	58.13	0.354506	0.123076	0.439144	3.568072
Solution 3	1	41.22	0.142639	0.081734	0.224373	2.745161
	3	51.07	0.304302	0.083602	0.387904	4.639889
	5	60.07	0.287079	0.120576	0.407655	3.380897

As a consequence of the process mass of the samples was reduced. The highest value of WR parameter (0.354506 g/g i. s. w.) was obtained after 5h in solution 2.

SG value shows the degree of penetration of solids from hypertonic solution into the meat samples. SG, during osmotic dehydration of pork meat, showed a tendency to increase with increasing the immersion time. The lowest value of SG parameter after five hours of process was obtained in samples dehydrated in solution 3.

High value of WL/SG ratio is the most important indicator of the effectiveness of OD treatment (Lević at al., 2007). The highest value of the WL/SG (4.639889 g/g i.s.w) was achieved in by using sugar beet molasses as osmotic solution after 3h of the treatment.

Increasing the dehydration time causes a greater water loss from meat samples. The highest WL value (0.439144 g/g i.s.w.) was noticed in samples dehydrated 5h in solution 2.

Changes of NMC and NSC parameters for the meat samples immersed in three different osmotic solutions at 20^{o}C for 5h are shown in fig 1.

During first 3 hours of OD intensive decrease of NMC and at the same time increase of NSC parameter occurred for all three solutions. During this time water removal was about 50% of initial moisture content. After third hour of OD, the water and solid diffusion slowed down. According to these results the time of dehydration can be limited to 3 hours.

The lowest value of NMC parameter was obtained in samples dehydrated in solution 2, after five hours of dehydration (0,384). Comparing results for NSC parameter for three solutions, samples dehydrated in solution 3 at the end of process had the lowest value (1.446). This makes molasses desirable as osmotic agent, considering that the aim of dehydration, besides obtaining the highest water loss, is to avoid great penetration of solids into the sample (Mišljenović at al., 2011).

The overall mass transfer coefficients for water and solute are shown in fig. 2 and 3. These coefficients were studied as a function of immersion time and different type of osmotic medium.

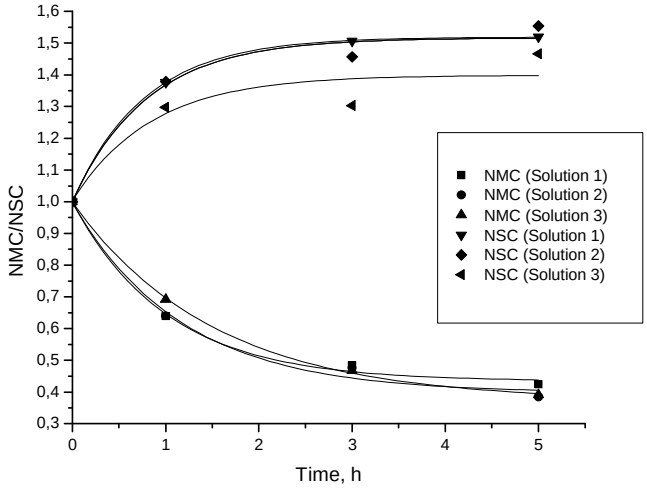

Fig 1. Influence of different osmotic media and dehydration time on the NMC and NSC parameters

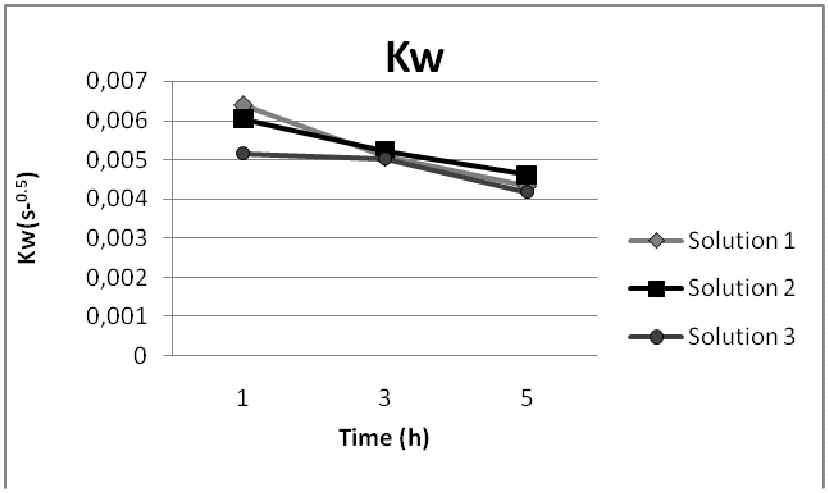

Fig.2**.** Mass transfer coefficient for water during osmotic dehidration of pork meat in three different osmotic solutions at 20^{o}C

Mass transfer coefficients decreased with duration of the treatment. Higher values of mass transfer coefficients for water at the first 3 hour of process can be explained by a greater driving force at the beginning of the process.

Comparing values of mass transfer coefficients obtained for three osmotic solutions it can be noticed, that all three solutions are efficient osmotic mediums, considering that Kw values were similar after 3h of OD.

As mentioned, great solid uptake should be avoided during OD. Ks values were similar for first two solutions, but solution 3 gave lower values. This also goes in favor to sugar beet molasses being convenient agent for OD.

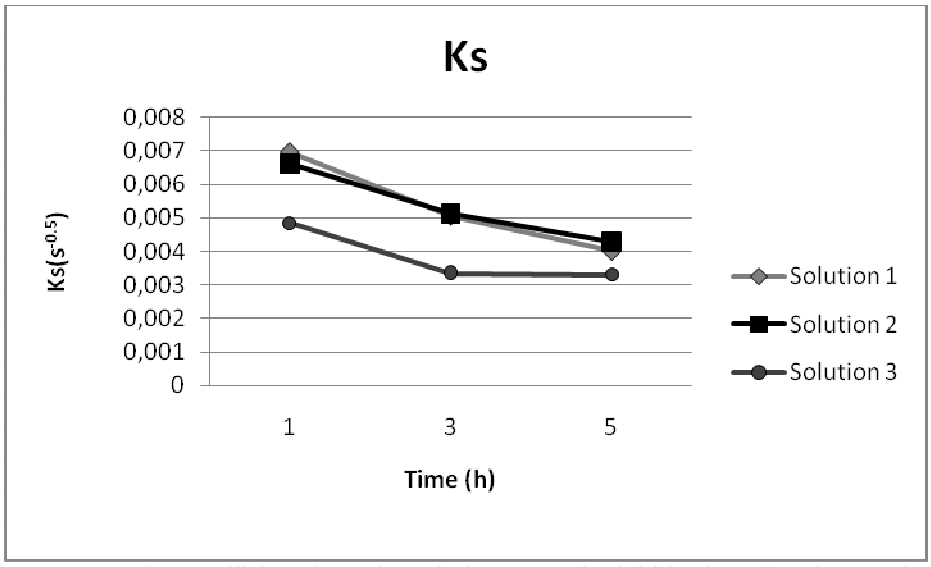

Fig.3. Mass transfer coefficient for solute during osmotic dehidration of pork meat in three different osmotic solutions at 20^{o}C

CONCLUSIONS

Considering obtained results it can be concluded that all three solutions are satisfying osmotic agents. The best results regarding dry matter content, weight reduction, solid gain and *WL*/*SG* ratio were achieved using sugar beet molasses as osmotic agent, which is economy reasonable considering that molasses is side product of sugar industry. During osmotic dehydration of pork in all three osmotic solutions, water removing process was most intensive at the beginning and after 3 hours had tendency of stabilization, therefore processing time can be limited to 3 hours.

ACKNOWLEDGEMENTS

These results are part of project supported by the Ministry of Education and Science of the Republic of Serbia, TR-31055, 2011-2014.

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PHYSICAL AND SENSORY PROPERTIES OF CHEWING GUMS PREPARED WITH VARIOUS SWEETENERS

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ABSTRACT: Chewing gum is one of the most popular confectionery products, due to its sensory properties and possible health effects, such as positive influence on dental health, reduction of the post-operative ileus following gastrointestinal surgery, reduction of muscular tension and relief of gastroesophageal reflux disease symptoms. Different chewing gum recipes were designed in order to study influence of carbohydrate ingredients and commercial aroma preparations on sensory and textural properties of final product. Chewing gum samples were produced in the lab scale sigmoid kneader (Heligear HDMO) coupled with Thermomix 1480 thermostat. Sensory analysis was performed by evaluation of basic chewing gum properties (smell, taste, appearance, texture), together with hardness at the beginning of chewing, bubble size and gum extensibility. Instrumental texture analysis was measured with Stable Micro Systems Texture Analyser TA.HD.plus with 4 mm cylinder stainless steel probe. Texture analysis revealed lemon chewing gum with glucose syrup (DE=38) as the hardest of produced samples. Orange flavoured chewing gum produced with the same syrup was the softest. Sensory analysis results showed fructose syrup chewing gum being the most acceptable. Both sensory and instrumental texture analysis methods are only partly comparable.
Keywords: *chewing gum, sensory analysis, texture measurement*

INTRODUCTION

Chewing gum has been one of the most popular confectionery products for over one and half century but it is not very well known that chewing gum history is more than 10000 years old. The oldest evidence of people chewing gum was found in Huseby Klev in Sweden. The found chewing gum was from around the year 8000. BC (Kjellström et al., 2010). The popular history of chewing gum began in 1848. when John B. Curtis produced State of Main Pure Spruce Gum, which was made from spruce resin (Lee, 2001). But probably the most famous chewing gum producer was William Wrigley Jr, who started selling Juicy Fruit and Wrigley's Spearmint chewing gum in the year 1893 (Matthews and Schultz, 2009).
Chewing gum mainly consists of the following main ingredients: gum base, carbohydrate or some other sweetener syrup, different bulk sugars and sweeteners, moisturisers, softeners, different flavours and colours and organic acids. Different ingredients have different influence on sensory and textural properties. Since gum base is one of the most important chewing gum ingredients, it is very important to choose suitable gum base. It has also been shown that dextrose equivalent of glucose syrup is very important. The most recommendable is glucose syrup with DE 38-44, while higher DE are responsible for stickier chewing gums which can be too soft (Howling and Jackson, 2005). The most frequently used bulk sweetener in chewing gums is sucrose. Although it has pleasant taste, it can be problematic because of hygroscopicity under unsuitable process conditions, which leads to crystallisation and formation of lumps. This problem may also occur with syrups, and the higher DE, the higher will be the rate of moisture pickup (Hull, 2010). Flavour is

the chewing gum ingredient which can be found in very small content but it has very important influence on sensory properties of chewing gum, as well as on its textural properties because of gum base and flavour interactions. Different flavours have different influence on the softness of gum base and chewing gum in general. Therefore, if flavour doesn't soften gum base enough, softer gum base must be used (Wright, 2010).

Many positive health effects of chewing gum have been discovered and many scientific research deal with that topic. It has been showed that chewing gum may limit the increase in daytime sleepiness (Johnson et al., 2012), may reduce postoperative ileus (Noble et al., 2012), may alleviate negative mood and reduce cortisol (Scholey et al., 2009), may suppress hunger, appetite and cravings for snacks and promote fullness (Hetherington and Reagan, 2011). However, scientific activity performed on physical properties of chewing gum, as well as their production procedures, has been very scarce. Therefore the aim of this study was to investigate the influence of different ingredients on the sensory and textural properties of chewing gums.

MATERIAL AND METHODS

Materials

Different chewing gum recipes were designed in order to study influence of carbohydrate ingredients and commercial aroma preparations on sensory and textural properties of final product. The following materials were used for the production of seven different chewing gums: gum base, glucose syrup DE=60 (Cargill, Germany), powdered glucose syrup DE=60 (Cargill, Germany), fructose syrup (Cargill, Germany), sorbitol syrup (Cargill, Germany), sucrose, fructose (dm-drogerie markt, Germany), powdered citric acid, glycerol (Prima Pharme, Croatia), powdered flavours of lemon, strawberry and sour cherry (Ireks Aroma d.o.o., Croatia), liquid flavours of blueberry, pear, orange, strawberry and acerola (Symrise GmbH&Co.KG, Germany), powdered colours (beetroot, strawberry, apocarotenal) and liquid colours (yellow E 102 and red E 122; AROMAR d.o.o., Croatia). All the mentioned materials were added in their primary state, except powdered glucose syrup DE=38 and powdered colours. Powder glucose syrup was diluted in water to form 80% solution. Powdered colours were diluted in few drops of water. Developed chewing gum recipes can be seen in table 1.

Table 1: Chewing gum recipes

Flavour type	Blueberry	Pear	Orange	Lemon	Strawberry	Acerola	Sour Cherry
Gum base (%)	19,76	19,87	20,02	19,96	19,11	19,79	16,99
Glucose syrup DE=38 (%)	-	-	18,02	17,97	-	29,69	-
Glucose syrup DE=60 (%)	17,78	-	-	-	-	-	-
Fructose syrup (%)	-	17,88	-	-	-	-	-
Sorbitol syrup (%)	-	-	-	-	17,19	-	14,39
Sucrose (%)	60,09	60,44	60,06	59,89	62,10	47,84	-
Fructose (%)	-	-	-	-	-	-	67,99
Citric acid (%)	0,49	0,49	0,66	0,99	0,47	0,49	0,53
Glycerol (%)	0,49	0,49	0,50	0,49	0,47	1,32	0,53
Colour (%)	0,25	0,09	0,17	0,21	0,03	0,25	0,14
Flavour (%)	1,14	0,72	0,57	0,47	0,21	0,62	0,39

Chewing gum production
Chewing gums were produced in the lab scale sigmoid kneader (Heligear HDMO) coupled with Thermomix 1480 thermostat. Gum base was melted in kneader at temperature between 65 and 70 ℃. Different ingredi ents were added to the gum base after melting in the following order: syrup, first half of sugar (or other bulk sweetener) and citric acid, second half of sugar (or bulk sweetener), softener, colour and flavour. Materials were added in intervals of around 5 minutes and the whole production process lasted 30-40 minutes. Chewing gum was then removed from the kneader and, after 10-15 minutes of cooling, cut into pieces.

Sensory evaluation
Sensory analysis was performed by evaluation of basic chewing gum properties (smell, taste, appearance, texture), together with bubble size and gum extensibility. Sensory analysis was performed by trained panel. Ten panellists were grading chewing gum smell, taste, appearance, texture, bubble size and extensibility. Smell, taste, appearance and texture were graded with scores from 0 to 10, with 10 being the highest score and 0 being the lowest. Bubble size was graded from 0-3 (0 - it is not possible to make a bubble, 1 - small bubble, 2 - medium bubble, 3 - big bubble). Extensibility was measured by stretching chewing gum with one's hand from teeth until the maximum extensibility of 100 cm. Bubble size and extensibility were measured in the first, sixth and eleventh minute of chewing. Chewing gum hardness at the beginning of chewing was also graded in order to compare it with instrumental analysis hardness results. The scores were also from 0 to 10, with 0 being very hard and 10 being very soft (Delarue and Loescher, 2004; McGowan and Lee 2006).

Texture analysis
Instrumental texture of chewing gum was measured with TA.HDPlus texture analyser (Stable Micro Systems, Great Britain) by samples penetration with 4 mm cylinder stainless steel probe. Chewing gum samples had the following dimensions: 20 x 20 x 5 mm, test speed was 1 mm/s and distance was 2mm (Bourne, 2002).

RESULTS AND DISCUSSION

When comparing total sensory evaluation results of chewing gum in table 2, it can be seen that chewing gums flavoured by pear (25,4), orange (25,3) and strawberry (24,5) received the highest scores. Pear flavoured gum received the highest texture score (7,9) and very high scores for taste (6,6) and appearance (7,5). Although it received lowest grade for smell (3,4), that didn't have very big influence on reaching the first place when it comes to overall acceptance of chewing gum. This gum had very pleasant aroma and adequate sweetness, together with prolonged soft texture. Although orange flavoured gum received the highest scores when it comes to taste (7,0) and smell (9,2), it received very low score for texture (2,5). The problem was stickiness of orange chewing gum, caused by the addition of higher content of aroma. That caused the gum to stick to teeth which made it almost impossible to chew. Strawberry flavoured gum received good scores for appearance and smell but the gum was not very well accepted, which can be seen from low scores for taste and texture. The three mentioned chewing gums had the same content of sucrose (around 60%) and gum base (around 20%), but different content of syrups. Therefore, it can be noticed that fructose syrup in pear and glucose syrup (DE=30) in orange flavoured gums give products with acceptable sweetness and texture properties, while the same cannot be said for the strawberry recipe which contained sorbitol syrup. Strawberry gum was very crumbly at the beginning of chewing, probably because of the crystallisation of main chewing gum ingredients. Crumbling at the beginning of chewing led to swallowing of main part of bulk sweetener and

aroma, which caused hardness of chewing gum with aroma which didn't last very long.

Table 2: Average scores of sensory evaluation of appearance, taste, texture and smell

Sample	Appearance	Taste	Texture	Smell	TOTAL
Blueberry	4,6±1,6	4,5±1,2	2,3±2,1	7,5±1,3	18,9
Pear	7,5±2,0	6,6±0,9	7,9±0,8	3,4±2,7	25,4
Orange	6,6±1,4	7,0±1,3	2,5±2,3	9,2±1,2	25,3
Lemon	8,7±1,6	6,0±1,3	5,3±2,4	3,7±2,1	23,7
Strawberry	8,7±1,0	4,6±1,4	3,4±2,1	7,8±1,9	24,5
Acerola	4,2±1,6	5,2±1,4	4,2±2,1	6,8±1,8	20,4
Sour Cherry	6,9±2,1	0,5±0,5	0,2±0,4	6,1±2,1	13,7

Chewing gums flavoured by sour cherry, blueberry, acerola and lemon received the lowest total scores, with sour cherry flavoured gum as the worst one. The problem of this gum was crumbling at the beginning of chewing, which led to hard texture and poor aroma of the gum. Therefore, combination of sorbitol syrup (15%) and fructose as bulk sweetener (68%) with gum base (17%) showed to be non satisfactory. The problem of blueberry flavoured chewing gum was inadequate syrup (glucose syrup DE=60), which made the chewing gum too hard and crumbly at the beginning of chewing. Also, the colour from beetroot showed to be inadequate, while the chewing gum completely lost the colour after the production process. The problem can be betalain, beetroot colour component, which can have lower stability in the presence of oxygen, light and increased temperature (Azeredo, 2009). Acerola flavoured chewing gum was also very crumbly because of higher glucose syrup content (30%) and lower sucrose content (48%). Higher moisture content of the chewing gum led to crystallization of main ingredients and crumbly texture at the beginning of chewing gum, which caused the loss of aroma and very hard texture during chewing. Lemon flavoured chewing gum didn't show any crumbling at the beginning of chewing but it was very hard throughout the whole chewing period. The reason may be lower moisture content because of the use of powdered aroma.

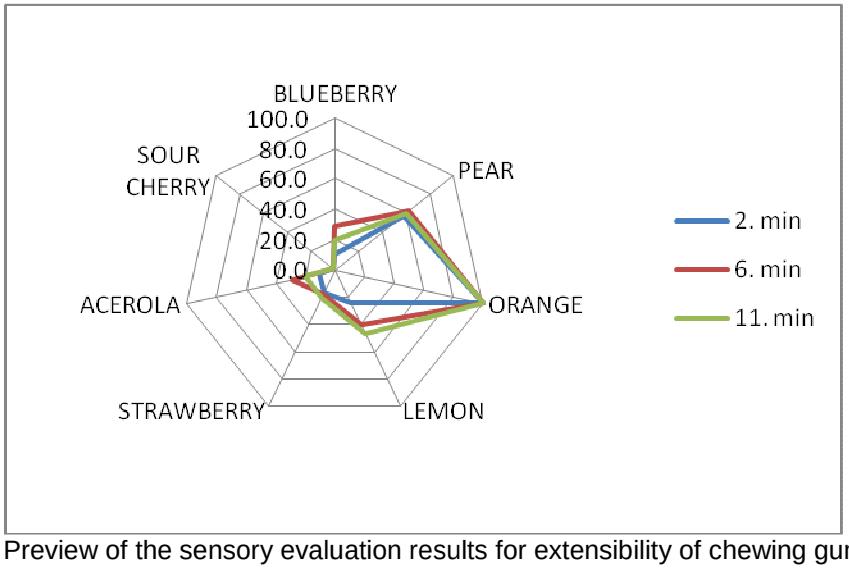

Figure 1: Preview of the sensory evaluation results for extensibility of chewing gum (in cm)

The most extensible chewing gum was orange with maximum extensibility during the whole period of chewing (100 cm), which can be seen in figure 1. It was also shown that pear and lemon flavoured chewing gums showed reasonable extensibility, while chewing gums flavoured by blueberry, acerola, strawberry and sour cherry didn't show reasonable extensibility. It can be seen that all chewing gums have the lowest extensibility at the second minute of chewing. The extensibility grows during chewing

period and reaches the highest point at the sixth minute, after which it starts to fall down until the eleventh minute of chewing.

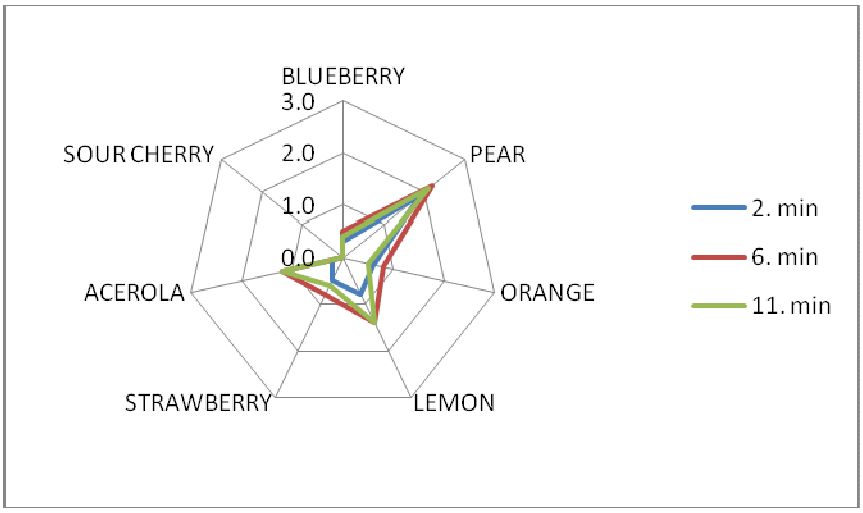

Figure 2: Preview of the sensory evaluation results for bubble gum size of chewing gum

The results in figure 2 showed that it is possible to make the biggest bubbles with pear flavoured chewing gum. While testing was performed during 11 minutes of chewing, it was shown that at the second minute of chewing it is possible to make small bubbles, but after 6 or 11 minutes it is possible to make medium bubbles. The results also showed that it was possible to make small bubbles with lemon and acerola flavoured chewing gums after 6 and 11 minutes of chewing. It was very hard, or almost impossible to make bubbles with chewing gums flavoured by blueberry, strawberry and sour cherry because of the crumbling and hard texture of chewing gums. It was also very hard to make bubbles with orange flavoured chewing gum because of the stickiness of the chewing gum.

Table 3: Sensory evaluation of hardness of chewing gums at the beginning of chewing

	Blueberry	Pear	Orange	Lemon	Strawberry	Acerola	Sour Cherry
Hardness	8,4	8,5	7,9	2,0	8,3	8,0	8,9

Table 4: Results of the instrumental texture measurement with 4 mm cylinder probe

SAMPLE	HARDNESS (N)	ELASTICITY (mm)	CHEWING WORK (kg/s)
Blueberry	14,214±1,508	1,9432±0,092	4,595±0,358
Pear	7,084±0,493	2,947±0,156	4,569±0,536
Orange	0,926±0,055	0,041±0,041	0,995±0,054
Lemon	35,675±1,397	2,275±0,188	16,833±1,284
Strawberry	12,260±0,343	1,655±0,356	4,837±0,686
Acerola	2,360±0,240	4,639±0,277	1,586±0,159
Sour Cherry	30,076±1,720	0,688±0,157	5,587±0,607

Instrumental texture analysis results from table 4 showed that lemon flavoured chewing gum (35, 675 N) was the hardest gum, followed by sour cherry (30,076 N), blueberry (14,214 N) and strawberry (12,260 N). The softest chewing gums were orange (0,926 N), acerola (2,360 N) and pear (7,084 N). Results of sensory evaluation of hardness (table 3) also showed that lemon flavoured gum was the hardest product, but all other chewing gums were graded as very soft and received scores between 7.9 and 8,9. This shows that instrumental and sensory analyses of hardness cannot be compared in this case. The reason may be that most of the chewing gums were crumbly at the beginning. While the texture analyzer recognized

carbohydrate crystals in chewing gums reasonably hard, panellists experienced those chewing gums as very soft in the mouth.
The results for chewing work can be compared to hardness results, while the highest chewing work is needed for the hardest chewing gums. Therefore the highest chewing work was measured for lemon flavoured chewing gum, followed by sour cherry, strawberry and blueberry. The lowest chewing work was measured for gums flavoured by orange, acerola and pear.
Instrumental texture analysis also showed acerola flavoured gum as the most elastic chewing gum, followed by pear, lemon, blueberry and strawberry gums. The least elastic chewing gums were sour cherry and orange. The results in this case are very confusing while orange chewing gum, when stretched with hands, was the only gum showing any elasticity at all. All other chewing gums, except pear and lemon flavoured gums, showed no elasticity and they would instantly break.

CONCLUSION

Based on the results of sensory evaluation it can be concluded that the most acceptable chewing gum recipe contains gum base (20%), fructose syrup (18%) and sucrose (60%) with addition of low content of flavour, colour and glycerol. Glucose syrup with DE=60 is not acceptable for chewing gum production because of the hardness of chewing gums. Glucose syrup with DE=38 is more acceptable because chewing gums are softer. However, recipes with glucose syrup DE=38 (18%), gum base (20%) and sucrose (60%) are not acceptable because of the chewing gum crumbling at the beginning of chewing, and consequentially, hardness and flavour loss. Orange flavour content of 0,6% is not acceptable in chewing gum recipes because of the chewing gum stickiness. Instrumental texture analysis of hardness and sensory analysis of hardness cannot be correlated precisely. The problem occurs with chewing gums which show signs of crystallization, while texture analyser recognizes them as hard, and panellists recognize them as soft.

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POSSIBILITIES FOR THE USE OF PLANT OILS IN FERMENTED SAUSAGES PRODUCTION

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ABSTRACT: Three batches of fermented sausages were made: control (C – 75/25 meat/backfat ratio) and two variants (20% of backfat was substituted by grapeseed oil): variant 1 (var1) with pre-emulsified oil and variant 2 (var2) with encapsulated oil as microspheres. pH (days 2, 7 and 15), basic chemical composition (days 0 and 15), colour, texture profile analysis (TPA) and sensory evaluation (day 15) were monitored. Single-factor analysis of variance was used ($P<0.05$). Changes of pH value were similar in all variants, reaching minimum 5.08–5.16 (day 2) and values 5.21–5.35 (day 15). As for the basic chemical composition, there was a significant difference in the moisture content between variants C and var1 with respect to var2 (day 15). Colour measurements showed a significant difference in terms of lightness (L* – var1 and var2), redness (a* – var2 and C), yellowness (b* – in all variants). As for TPA, the most pronounced differences were observed in terms of hardness and chewiness; the highest were in var1, 2–4 times greater when compared to C and var2. Sensory evaluation showed no differences in terms of appearance, cut appearance or colour. In terms of odour and taste, the best graded was var2. The texture of var1 was graded significantly lower than the other two variants. Backfat can be substituted with oil prepared as illustrated in the experiment, though with certain corrections primarily because of texture. More research is required in order to monitor the changes in observed parameters during storage.
Key words: *fermented sausage, grapeseed oil, encapsulated oil, CIE L*a*b*, Texture profile analysis*

INTRODUCTION

Over the past years the demand for functional food has increased because nowadays the purpose of food is not only to satisfy hunger but also to prevent nutrition-related diseases and improve the physical and mental well-being of consumers (Siró et al., 2008). Apart from the basic nutrients, functional food should contain a component with a selective effect on one or various functions of the organism whose positive effects can be justified as functional (physiological) or even healthy (Zhang et al., 2010). Japan is the first country that developed the idea of functional foods (in 1980s) and has established regulations for the use of functional foods (Siró et al., 2008; Zhang et al., 2010).

Meat and meat products are important sources of proteins, fat, essential amino acids, minerals and vitamins and other nutrients (Vuković et al., 2009; Zhang et al., 2010). On the other hand, it seems that there is a relationship between a high-fat intake, especially of saturated fat, and an increased risk of some cancers (especially colon, breast and prostate cancer) and coronary heart diseases (Chizzolini et al., 1999; Rubio et al., 2008).

Fermented sausages have been manufactured for centuries. They are even mentioned in historic records of ancient Greeks, Romans and the Chinese (Zeuthen, 2007). For years these products were perceived as a significant source of proteins of

high biological and caloric value. Nowadays, with changes in the diet, as well as development of new and perfection of conventional preservation methods, fermented sausages are more highly appreciated because of their sensory characteristics.
In the sense of functional products, requirements for meat and meat products are reflected in the reduced level of fat, cholesterol, decreased contents of sodium chloride and nitrite, improved composition of fatty acid profile and incorporated bioactive compounds (Zhang et al., 2010). In the case of cured products, new formulations have facilitated the production of healthier dry fermented sausages by minimizing two of their major drawbacks: high salt content and the presence of saturated fatty acids (SFA) and cholesterol (Valencia et al., 2006). Improvement of functional properties of fermented sausages in the sense of changing the composition of fatty acids is reflected in the change of PUFA/SFA and ω-6/ω-3 ratios, which are the parameters for assessing the nutritional quality of the lipid fraction of food. This can be achieved by substituting one part of backfat with oils rich in unsaturated fatty acids, such as olive oil, canola oil, fish oil, linseed oil, grapeseed oil, etc. Bearing in mind that fat contributes to the colour, texture and flavour of fermented sausages, the addition of these oils makes sense only if the sensory quality is either not altered or slightly changed, because otherwise the acceptability of a product would be reduced.
The objective of this research was to establish the extent of changes of colour, texture and sensory characteristics of dry fermented sausages with the addition of grapeseed oil.

MATERIAL AND METHODS

Sausages manufacture and oil preparation

Three batches of dry fermented sausages were made. Pork meat and backfat were used in the ratio of 75:25 to produce control variant (C). Other two variants were made by substituting 20% of backfat with grapeseed oil: variant 1 (var1) with pre-emulsified oil and variant 2 (var2) with encapsulated oil. Meat and fatty tissue were ground in a cutter to about 5 mm and equal amounts of ingredients were added to all sausage variants: 2.5% salt, 0.011% $NaNO_2$, 0.2% dextrose, 0.15% sucrose, 0.04% sodium erythorbate, 0.20% garlic, 0.3% powdered white pepper and commercial starter culture mixture (*Lactobacillus curvatus*, *Staphylococcus carnosus* and *Staphylococcus xylosus*). Collagen casings (32 mm diameter) were used for filling. The ripening took place in a drying chamber under controlled conditions and under the following regime: day 1 – relative humidity (RH) 92% at 22 ℃, day 2 RH 90% at 19 ℃ with 6 h of smoking, day 3 RH 88% at 17 ℃ wi th 6 h of smoking; during the following days RH was reduced daily by 1% at 16 ℃. The production lasted 15 days.
Pre-emulsified oil (var1) was prepared by mixing for 2 min 5 parts of tap water with one part of soy protein isolate (SPI) (Solae, LCC) and then with 5 parts of grapeseed oil for another 3 min. Encapsulated oil (var2) was prepared in the following manner: alginate powder was dispersed in distilled water to produce solutions of 0.02 g/ml. The oil/alginate emulsion (40% w/w oil and 60% w/w alginate solution) was prepared using Ultra-Turrax T25 (T25 digital ULTRA-TURRAX®, IKA, Germany) at the speed of 10000 rpm for 5 min. Alginate/oil emulsion was extruded through 0.7 mm blunt stainless still needle using a syringe pump (Pump 11, Harvard Apparatus, SAD) under constant flow rate of 70 ml/h. The spherical droplets were formed by combined action of electrostatic force and gravity. Electrostatic potential (6.5 kV) was formed by electrostatic encapsulation unit (Nisco Encapsulator, Switzerland). The collecting solution was calcium chloride (0.015 g/ml). The distance between the needle tip and the collecting solution was 2.5 cm. After extrusion, the beads were left in the collecting solution for 45 min. After that, the beads were rinsed and left in distilled water at 4 ℃.

Methods

Three sausages were randomly taken from each group for pH, chemical analysis, texture profile analysis (TPA) and colour measurements.

On days 0 and 15 six individual sausages were weighed on the scales (Chyo MK-2000B) with a 0.1 g precision ratio, in order to determine weight loss. The chemical composition was determined in the following manner: water content by drying samples at 105 ℃ (ISO 1442, 1997); protein content by the Kjeldahl method and multiplying by factor 6.25 (ISO 937, 1978); fat content by the Soxhlet method (ISO 1443, 1973). Chemical analysis was conducted at the beginning (day 0) and the end (day 15) of production. pH value was measured by pH-meter Hanna, HI 83141 (Hanna Instruments USA) on days 0, 2, 7 and 15.

Sausage colour was determined by Chromameter CR-400 (Minolta Co. Ltd, Tokyo, Japan), in line with the CIE L*a*b* system (L* – lightness, a* – redness, b* – yellowness), on day 15. The colour of the cross section was determined immediately after the samples were cut and measurements were performed at room temperature (20±2 ℃). Texture profile analysis (TPA) was perfo rmed using the universal texture analyzer TAXP (Stable Micro System, Godalming, England). Samples were taken from the centre of the sausage, 2 cm in height and 2.54 cm in radius, held for equilibration to room temperature and compressed twice to their original height, with a compression aluminium platen of 75 mm (P/75) and a 250 kg load cell. Pre-test speed was 3 mm/s, test speed was 1 mm/s and post-test speed was 1 mm/s. Hardness, adhesiveness, springiness, cohesiveness, and chewiness were evaluated using the available computer software.

The evaluation of sensory characteristics of sausages was conducted at the end of production (15 day) by eight assessors. A numeric-descriptive scale with a nine-point system was used to evaluate the appearance, cross section, colour, odour, texture and taste of sausages (1 – extremely unacceptable, 9 – extremely acceptable).

The results were processed by single-factor analysis of variance (ANOVA). The differences between individual averages were tested using Tukey's method. Significant differences were considered for $P<0.05$. Calculations were done with software Statistica 6.0 PL, for Windows (Statsoft Inc.).

RESULTS AND DISCUSSION

The basic chemical composition of all three variants was very similar (Table 1). The variants with added oil had higher initial moisture content, but the difference is not significant. Consequently, their protein contents were lower, but this difference is also not significant. At the end production the weight loss of variants var1 and var2 was significantly higher than that of the control variant (40.47%, 39.87% and 37.94%), but the water content was significantly higher only in var2, most likely due to the water added through emulsion (var1) or microspheres (var2). Bloukas et al. (1997) found that the partial replacement of pork backfat with olive oil in the form of pre-emulsified fat with SPI resulted in lower moisture content and higher weight loss than control, and that the higher the incorporated emulsion, the higher the weight loss.

Table 1. Basic chemical composition

		C	Var1	Var2
moisture	Day 0	55.86±0.72^{a}	57.10±0.92^{a}	57.41±2.18^{a}
	Day 15	28.19±0.10^{a}	28.06±0.85^{a}	30.33±0.13^{b}
fat	Day 0	24.51±1.59^{a}	24.10±1.85^{a}	24.04±0.43^{a}
	Day 15	40.52±1.29^{a}	40.79±1.55^{a}	40.19±2.14^{a}
proteins	Day 0	15.21±0.73^{a}	15.22±0.16^{a}	14.61±0.98^{a}
	Day 15	24.06±1.09^{a}	25.52±0.91^{a}	23.61±1.03^{a}

Results are given as mean ± standard deviation (n = 3). Different superscripts in the same row for the same day indicate significant difference (P<0.05)

Muguerza et al. (2001) found that treatments with 20 and 30% replacing level of pork backfat with pre-emulsified olive oil (with SPI) showed higher water content than control. Kayaardi and Gok (2004) had lower weight loss in variants with added oil in the form of pre-emulsified fat (with SPI) when compared to control, but with no significant differences in terms of moisture content.
The change in pH values during production was of a similar pattern (Figure 1), with the minimum achieved on day 2, followed by a slight growth until production end. Kayaardi and Gok (2004) and Bloukas et al. (1997) emphasised that the addition of olive oil as soy emulsion has no effect on pH.

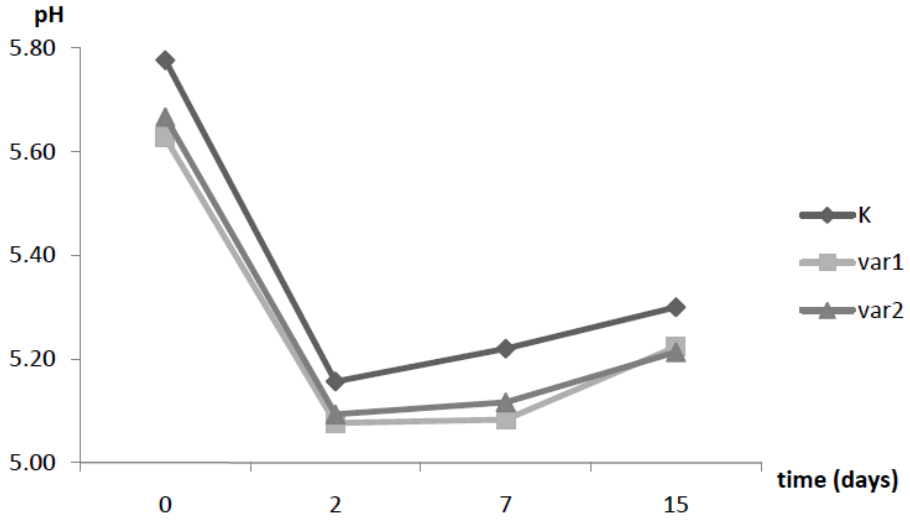

Figure 1. Changes of pH values during ripening

Colour is a very important quality characteristic of fermented sausages, since it influences consumer acceptability (Bozkurt and Bayram, 2006). Results of colour measurements are shown in table 2.

Table 2.Colour measurements

	C	Var1	Var2
L*	50.99±2.58^{ab}	52.23±0.91^{b}	49.05±1.30^{a}
a*	11.57±0.88^{a}	12.27±1.01^{ab}	12.99±0.82^{b}
b*	6.16±0.62^{a}	8.23±1.14^{b}	7.20±0.59^{c}

Results are given as mean ± standard deviation (n = 9). Different superscripts in the same row indicate significant difference (P<0.05)

Lightness measured in var1 was higher, and in var2 lower than in control, but the observed difference is not significant. Pelser et al. (2007) did not determine any higher values for L* in variants with added emulsified oil and encapsulated oil in comparison with the control variant. Muguerza et al. (2002) established that by replacing 20% of backfat with pre-emulsified olive oil in sausages with a higher initial backfat content, it is possible to create a lighter product. Bloukas et al. (1997) obtained greater differences in L* value in variants with added oil in respect to control. The addition of oils as microspheres resulted in darker product colour. Redness (a*) was the highest in var2 and significantly higher only in comparison with C. Pelser et al. (2007) did not determine any significant differences between variants with added emulsified and encapsulated oils. Similar conclusions were drawn by Muguerza et al. (2002) and Bloukas et al. (1997) for products with added emulsified oil. The control variant was less yellow (b*) than the modified variants and a significant difference was observed in all three variants. Other authors (Bloukas et al., 1997; Kayaardi and Gok, 2004; Muguerza et al., 2002; Pelser et al., 2007) reached similar conclusions.

Bloukas et al. (1997) found that fermented sausages with incorporated olive oil as pre-emulsified fat (with SPI) were harder than the control. Muguerza et al. (2001) on the contrary reported a reduction in hardness and chewiness in variants with pre-emulsified olive oil. In our case (Table 3), hardness and chewiness were by far higher in var1 when compared to control, as well as when compared to var2. Moreover, hardness and chewiness of var2 were significantly lower when compared to the control variant. There is a mutual dependency between moisture content on the one hand and hardness and chewiness on the other (Bozkurt and Bayram, 2006), and it was higher in var2; however, since hardness and chewiness in C were by almost 50% higher than in var2, it is possible that this was also a result of the addition of encapsulated oil. Bozkurt and Bayram (2006) remarked that adhesiveness during ripening is reduced by decreasing the moisture content, which makes sucuk easily sliceable. Since in our case the moisture content at the end of production was significantly lower in C and var1 when compared to var2, we can expect var2 to stick to the knife during slicing more than variant C. Data on the application of encapsulated oils is scarce and it mostly refers to the application of encapsulated fish and linseed oil in powder form (Pelser et al., 2007; Josquin et al., 2012), a product different from the one we used.

Table 3. Texture profile analysis

	C	var1	var2
Hardness (g)	6694.81±835.20b	15316.91±1047.97c	4446.70±318.74a
Adhesiveness (g*s)	-322.37±111.60b	-201.74±99.86ab	-152.34±66.23a
Springiness (mm)	0.46±0.03a	0.51±0.02b	0.48±0.02ab
Cohesiveness	0.51±0.02b	0.48±0.01a	0.46±0.02a
Chewiness (g*mm)	1583.38±246.19c	3700.22±359.09b	987.92±73.10a

Results are given as mean ± standard deviation (n = 6). Different superscripts in the same row indicate significant difference (P<0.05)

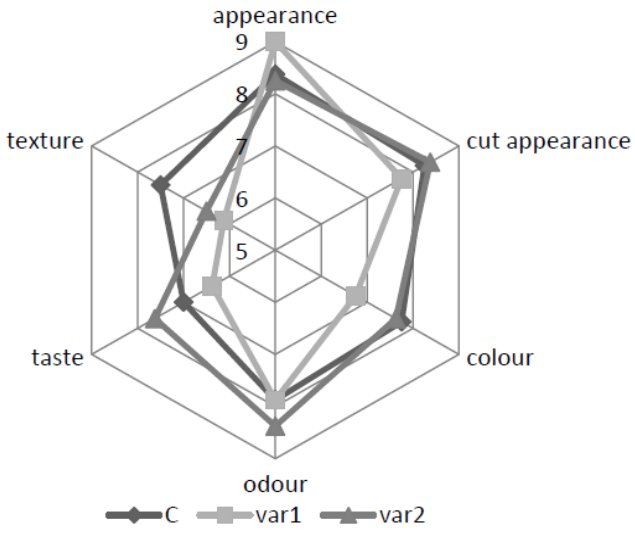

Figure 2. Sensory evaluation

Sensory evaluation (Figure 2) did not yield any significant differences between control and the variants with added oil. Assessors did not observe any significant differences in terms of appearance, cut appearance, colour and odour. In terms of taste there was only a difference between var1 and var2. Bloukas et al. (1997) reported similar results for fermented sausages with pre-emulsified olive oil added. The most pronounced differences were observed in terms of texture, where both var1 and var2 received grades lower than 7, but only var1 received a significantly lower grade in comparison with the control variant and var2. Since the measured values for hardness and chewiness in var1 were many times higher than in C and var2, it may

be possible that assessors perceived this as a flaw. Also, assessors gave a similar grade to texture in var2 in which significantly lower values of hardness and chewiness were measured, compared to C, as well as var1; however, this difference was not significant although texture received a grade lower than that of the control variant. It is possible that assessors perceive both too hard and too soft a texture as equally unfavourable. Kayaardi and Gok (2004) reported higher or equal marks for soudjouks prepared by replacing beef fat with olive oil in terms of colour, appearance, taste and texture than control.

CONCLUSIONS

The use of oil in ways described in the research is possible and it must be noted that the addition of oil did not render the product unusable. Variants with added oil had greater weight loss compared to the control variant, but the water content significantly differed only in var2. There were no significant differences in terms of protein and fat contents or pH values in all variants. Lightness significantly differed between var1 and var2, redness between C and var2, while yellowness differed between all three variants. As for texture profile analysis, the largest differences were observed in terms of hardness and chewiness, which were higher in var1 than in the other two variants. Assessors did not observe any significant differences in terms of appearance, cut appearance, colour and odour. Texture in var1 received the lowest grades, which may be related to the measured high values of hardness and chewiness. Grades of all sensory evaluation parameters were satisfactory (not lower than 6).
Further research should be focused on the changes of sensory characteristics during storage bearing in mind the shelf life of fermented sausages and tests to determine the fatty acid content and lipolytic changes.

ACKNOWLEDGEMENTS

Research was financed by the Ministry of Education and Science, Republic of Serbia, project III–46010. The authors thank Mr. Mladen Gavrilović (PALCO d.o.o., Šabac, Serbia) for alginate donation, Mr. Nenad Nikolić (Solae, LLC) for soy protein isolate donation, Mr. Mile Arnaut (Gram, Belgrade, Serbia) for calcium chloride (food grade) donation. We would also like to thank meat manufacturer PKB IMES Belgrade for their assistance with sausage production. We thank Marija Stajić for assistance with the translation.

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PREPARATION OF WATER-IN-OIL EMLUSIONS USING MEMBRANE EMULSIFICATION SYSTEM AND HIGH-SPEED HOMOGENIZER

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ABSTRACT: Water-in-oil emulsions, consisting of demineralized water dispersed in sunflower oil and stabilized with polyglycerol polyricinoleate, have been prepared by using two emulsification methods: high-speed homogenizer and ceramic membranes (alpha-aluminum oxide), with mean pore size 20 nm. Influences of the water phase content on the mean droplet size and droplet size distribution were investigated. The mean droplet size and droplet size distribution of the prepared emulsions were measured using a laser light scattering instrument. As expected, the results showed that the mean droplet size and droplet size distribution depended on the dispersed phase content in the emulsion in a way that increasing the water phase content the mean droplet size was increased. The results showed that application of membrane emulsification process can significantly reduce mean size diameter of water droplets in emulsions of this type. However, when membrane emulsification is used, the small water droplets, which were initially of the diameters less than 100 nm, tend to reduce the total amount of interface by coalescence, and form the droplets of the mean peak diameter of about 400 nm. At the same emulsifier content in the continuous phase (3%, w/w), the mean droplet diameter was about 750 nm in emulsions produced using high-speed homogenizer.
Key words: water-in-oil emulsions, sunflower oil, PGPR, membrane emulsification

INTRODUCTION

Production of water-in-oil (W/O) and water-in-oil-in-water (W/O/W) emulsions shows increasing interest in the food industry, due to the fact that within these emulsion systems different active ingredients can be captured, allowing they use in masking bad tasting or bad smelling components, controlled releasing of active ingredients and stabilization food ingredients.

Preparation of W/O emulsions requires the presence of certain amounts of lipophilic surfactant (low hydrophilic-lipophilic balance (HLB)), which reduces interfacial tension between two phases, prevents the occurrence of aggregation and increases emulsion stability (Van der Graaf at al., 2005). Previous studies showed (Surh et al., 2007) that polyglycerol polyricinoleate PGPR (HLB ≈ 3) possesses the optimal characteristics for obtaining W/O emulsion, because of relatively good solubility in oil and the possibility of the formation emulsion with droplets of small diameter and narrow droplet size distribution (Mun at al., 2010). The temperature of emulsification process did not show a meaningful effect on the droplet size and droplet size distribution (Surh et al., 2007).

Also, the concentration of surfactant affects the mean diameter of formed emulsion droplets, in the manner that droplet size decreases with increasing concentration of surfactant (Marquez at al., 2010). On the other hand, maximum *per capita* mean daily intake of PGPR is 2.64 mg/kg body weight/day (Wilson at al., 1998), by limiting the application of surfactant.

Size and droplet size distribution depends, besides the content of surfactant, on the dispersed phase content in the emulsion in a way that the water phase content increase increases the mean droplet size (Kobayashi at al., 2005).

Emulsions are usually prepared using high-speed homogenizers and rotor-stator systems, where the fine droplets are formed in the dispersing zone, under the influence of high shear stress. Development of new emulsification methods allows the production of emulsions with significantly improved characteristics (Joscelyne at al., 2000).

Membrane emulsification (ME) involves the permeation of pure dispersed phase through a microporous membrane into the continious phase flowing inside the membrane. Techniques to produce emulsions using membranes can be divided in two groups: either a coarse pre-mixed emulsion can be pressed through the membrane to reduce the droplet size of the disperse phase, or only the dispersed phase is pressed through the membrane forming droplets in the membrane surface that are detached by the cross-flowing continuous phase (Gejsbertsen-Abrahamse at al., 2004).

Previous studies have shown that cross-flow emulsification processes have significant advantages in the production of monodispersed emulsions, emulsions with shear sensitive components and nanometer-size emulsions and allow the preparation of stable emulsions with a very narrow droplet size distribution in comparison with conventional methods, such as homogenizers (Vladisavljević at al., 2002).

The aim of this study was to compare the emulsification processes and the properties of W/O emulsions prepared using membrane emulsification system (ME) and high-speed homogenizer. Also, content of dispersed phase in the emulsions and concentration of surfactant (PGPR) in the continuous phase on the size and droplet size distribution were investigated. This paper will emphasize influence of these two factors on the mean droplet diameter, the peak width and the span of droplet size distribution (PSD). The span of PSD is given by an equation:

$$span = \frac{d_{90} - d_{10}}{d_{50}}$$

where d_{x0} is the diameter corresponding to $x0$ vol.% on a relative cumulative PSD curve (Vladisavljević at al., 2003).

MATERIAL AND METHODS

For the preparation of the continuous phases sunflower oil (“Vital”, Vrbas, R. Serbia), and polyglycerol polyricinoleate (PGPR 90), kindly provided by “Jaffa” a.d. (Crvenka, R. Serbia), were used. Concentration of PGPR in the continuous phase (E) was 3 and 5 wt.% in both method of emulsification. Vegetable oil was used in its original form. Demineralized water was used as the dispersed phase.

Preparation of W/O emulsions using ME

The experimental set-up used for membrane emulsification is shown in Fig. 1. The ceramic $\alpha - Al_2O_3$ tubular membrane (250 mm length and 7 mm inner diameter), with the mean pore size of 20 nm, was placed in a membrane module (1) of the membrane emulsification apparatus. The continuous phase, prepared by dissolving a certain amount of PGPR (3 or 5%) in sunflower oil at 50℃, by mixing on a magnetic stirrer for 1/2 h, was forced to recirculate inside the system by using a peristaltic pump 620U (2) (Watson-Marlow, England). The continuous phase content in a tank (6) at the beginning of the process was 1500 ml, while the capacity of the dispersed phase graduated vessel (3) was 600 ml. The dispersed phase (water) was poured (7) into the pressure vessel (3) and introduced in the annular space of the module (1) by compressed nitrogen (N_2) from the gas cylinder (4). Water droplets formed in the membrane were detached from the membrane surface inside the tube by the cross-

flowing continuous phase. The continuous phase circuit worked in a closed loop. The produced emulsion was gradually enriched with the water phase with each run through the membrane tube. Samples of prepared emulsions were taken for the water phase contents of 10, 12.75, 15.50, 18.25 and 21% (w/w). Volumetric flow of the continuous phase was measured (5) and kept on 2.5 $L \cdot h^{-1}$. Transmembrane pressure was $0.9 \cdot 10^5$ Pa.

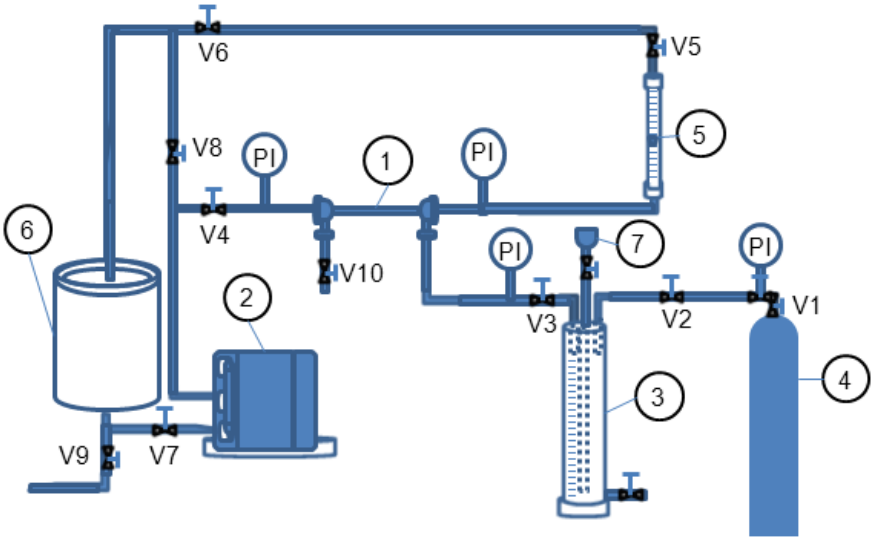

Figure 1. Membrane emulsification system: 1-membrane module, 2-peristaltic pump, 3-dispersed phase graduated vessel, 4-gas (N_2) cylinder, 5-flow meter, 6-continuous phase tank, 7-dispersed phase entrance, V1-10 – on-off valves.

Preparation of W/O emulsions using high-speed homogenizer

Continuous phase was prepared in the same way as in the previously described method, dissolving a certain amount of PGPR (3 or 5%) in sunflower oil at 50℃, by mixing on a magnetic stirrer for 1/2 h. The aqueous phase was dispersed drop by drop into the continuous oil phase with a continuous stirring by a high-speed homogenizer Ultra turrax T-25 (IKA, Germany) at 24 000 rpm for 5 min. Emulsification temperature was 25℃. The content of the disperse phase in the emulsions (W) was 21 wt. %.

Characterization of W/O emulsions

The mean droplet size and droplet size distribution of the water in W/O emulsions stabilized with PGPR was measured by laser light scattering instrument Zetasizer Nano ZS (Malvern Instruments, U.K.). The measurements were preformed immediately after the formation emulsions and repeated at least three times.

RESULTS AND DISCUSSION

The droplet size distribution properties of W/O emulsions prepared using ME system are shown in Figure 2. The effect of dispersed phase content on the size and droplet size distribution in the membrane emulsification process is shown in Figure 2a. The mean size diameter of water droplets (d) for the investigated water phase contents was in a range from 250 to 400 nm, with the peak width (pw) from 60 to 160 nm. As previous studies has shown the increase of the dispersed phase content leads to the increase of droplet diameter, nonetheless the investigated contents of the emulsifier. However, droplet size diameters were very similar to both concentration of PGPR, and when the content of dispersed phase was 21% (w/w) the diameters were almost the same of about 400 nm. The particle size distribution results depend also on the

storage time since the small water droplets, which were initially of the diameters less than 100 nm, tend to reduce the total amount of interface by coalescence, and form the droplets of the specified larger diameter within a short period of time after the emulsion production. The amount of surfactant needed for stabilisation of the interface depends on the amount of contact area between phases, which is significantly increased when such a small droplet diameters are produced by ME process. Therefore, the concentrations of emulsifier higher than 5% (w/w) are needed in order to prevent coalescence of small water droplets, and probably only these higher concentrations would show significant effect on droplet size diameter. But, PGPR content of 5% seems to reduce the peak width on the PSD curve when compared to the lower concentration of 3%. The same conclusion can be drown from the lower span values of PSD curve for 5% than for 3% of emulsifier, as it is shown in Figure 2b.

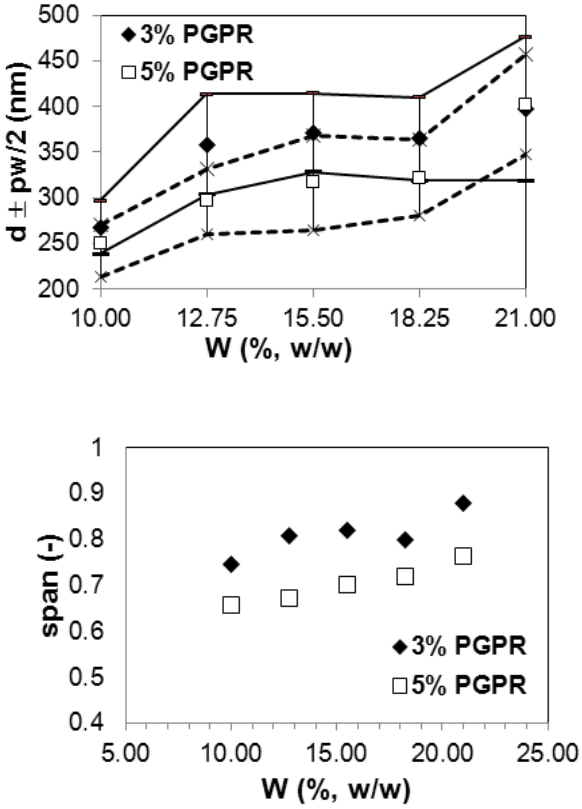

Figure 2. Results of the ME process as a function of dispersed phase content (W) for emulsifier contents of 3 wt.% (♦) and 5 wt.% (□); 2a) (on the left) The mean droplet diameter, d, (symbols) and the peak width values, pw, (lines) (symbols lie within d±pw/2 ranges placed between two solid lines (3 wt.% PGPR) or two dashed lines (5 wt.% PGPR). 2b) (on the right) The span of the particle size distribution curve.

W/O emulsions obtained by ME had a significantly smaller diameter of water droplets which was about 400 nm, for concentration of PGPR 3%, in comparison to the mean droplet diameter of emulsions obtained using homogenizer, which was about 750 nm, for the same dispersed phase content of 21 wt.% (Fig. 3a). As expected, increasing concentration of surfactant (5% PGPR) induced a decrease droplet diameter for emulsions obtained by homogenizer, but this behavior was not observed in the preparation of emulsions ME. This behavior can be explained by the fact that this is still very low concentration of surfactant for the ME process, taking into account that with decreasing droplet diameter increases the contact area between phases and therefore the greater amount of surfactant needed for interface

stabilization. However, the use of membranes, in a concentration of PGPR 5%, shows the reduction in the peak width on a relative cumulative PSD curve.
Membrane emulsification process demonstrated other one significant advantage in production W/O emulsions. Namely, the span of PSD curve of emulsions prepared in ME was considerably lower, especially for emulsions with 5% PGPR (Fig. 3b).

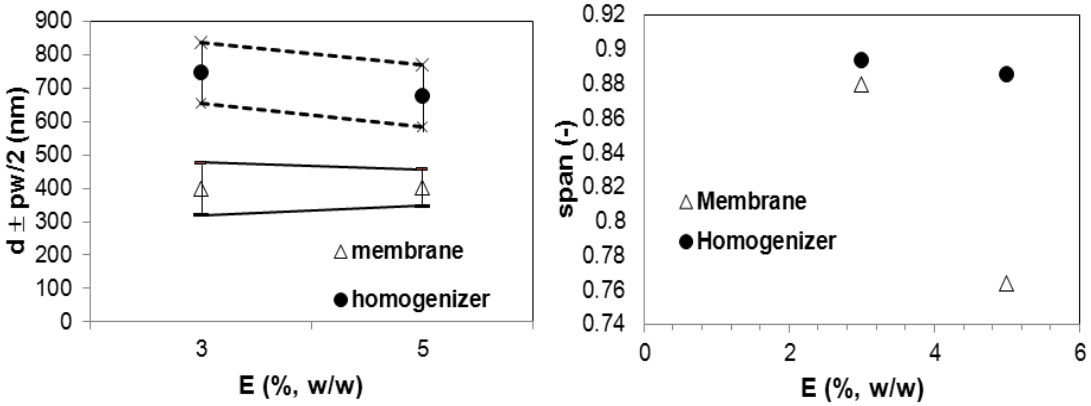

Figure 3. The particle size distribution properties of W/O emulsions with 21 wt.% of disperesed phase, prepared using membrane emulsification system (∆) and high-speed homogenizer (•) for the different contents of emulsifier. 3a) (on the left) The mean droplet diameter, d, (symbols) and the peak width values, pw, (lines) (symbols lie within d±pw/2 ranges placed between two solid lines (for ME) or two dashed lines (for homogenizer). 3b) (on the right) The span of the particle size distribution curve**.**

CONCLUSIONS

The mean size diameter of water droplets in W/O emulsions produced by membrane emulsification for the investigated water phase contents of 10, 12.75, 15.50, 18.25 and 21wt.% was in a range from 250 to 400 nm, with the peak width from 60 to 160 nm. By membrane emulsification process it is possible to produce W/O emulsions with a significantly smaller value of the mean water droplet diameter in comparison to the conventional emulsification process. For membrane and conventional processes, when the water phase content in the emulsion is 21 wt.%, and emulsifier content in the continuous phase is 3 wt.%, the mean droplet diameters are about 400 and 750 nm, respectively. For emulsifier content in the continuous phase of 5 wt.%, the span value of the particle size distribution curve of emulsions prepared in membrane emulsification process is considerably lower than corresponding values of emulsions obtained by homogenizer, 0.75 versa 0.90, respectively.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education and Science of the Republic of Serbia within the Integrated and Interdisciplinary Research Project No. 46010, 2011-2014.
We are grateful to the Department of Materials Engineering, Faculty of Technology, the University of Novi Sad, and to Professor Vladimir Srdić for providing us the possibility to characterise emulsions by using the instrument Zetasizer Nano ZS (Malvern Instruments, U.K.).

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SEPARATION OF PHOSPHOLIPIDS FROM PLANT MATERIAL RESIDUES

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ABSTRACT: Solubility of phospholipids in propane were measured in pressure range from 20 bar to 70 bar and temperature range from 40℃ to 60℃. Extraction of oils from pumpkin seeds were performed in the pressure range from 50 bar to 150 bar and at temperatures from 20℃ to 60℃. Oils produced by laboratory extractio n at those conditions were evaluated for phospholipid content and were round 6,5 wt.% independent from extraction pressure.
Key words: *lecithin, extraction, pumpkin seeds*

INTRODUCTION

Design of new products with special characteristics or design of new processes, which are environmental friendly and have an impact to sustainable processes, are a great challenge for chemical engineers. Supercritical fluids are relatively new tool, which leads to sustainable manufacturing methods that are not only ecologically preferable but also gives products with special properties. One of the latest applications is extraction of oil from plant materials to obtain highly concentrated and very pure lecithin. Separation and formulation of products by supercritical fluids and production of substances and composites with unique properties and characteristics for the use in different applications are now days intensively studied. One of the most important advantages of use of supercritical fluids is design of solvent free products with special product properties [Lütge et al, 2007]. Lecithin is a natural emulsifier, which is found in high concentrations in soy beans [Knez et al, 2010, King et al, 1994] and is a by-product of the soy bean oil production. In high content it can be found in some other seeds, like pumpkin seeds. It is used as a neutraceutical, as emulsifying agent in the food industry and as a source for phosphatidylcholine (PC) in the pharmaceutical industry. Lecithin is not a single substances but a mixture of different phospholipids: phosphatidylcholine (PC), phosphatidylethanolamin (PE), phosphatidylinositol (PI), phosphatoic acid (PA) and others. Pumpkin seeds contain beside highly valuable oils and beside being a good source of polyunsaturated fatty acids, such as myristic acid, palmitic acid, steric acid, oleic acid, linoleic acid, linolenic acid, arachidic acid etc., contain a lot of protein, zinc, and other vitamins, tryptophan and they are even said to lower cholesterol. Pumpkin seeds are a good source of magnesium, manganese, phosphorus, and phytosterols (Bernardo-Gil et al, 2004, Murković et al, 1996, Murković et al, 2000). In our research isolation of phospholipids and isolation of oils using propane were studied.

Figure 1: Pumpkin seeds.

MATERIAL AND METHODS

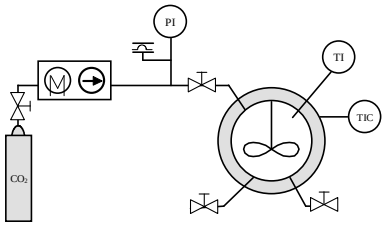
Materials

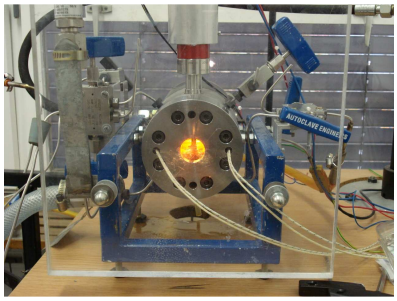
Propane with purity 3.5 was supplied by Messer (Ruše, Slovenia). Pumpkin seeds were supplied by Gea, d.d.(Slovenska Bistrica, Slovenia).Soybean lecithin (Sigma Aldrich) with 40% phosphatidylcholine was used for solubility studies.

Apparatus

High pressure view cell - Phase equilibria observation

For the process design solubility measurements and phase equilibrium observations were performed for the system lecithin (40% phospholipids)/propane in variable volume high pressure view cell was supplied by NWA (Lörrach – D) presented on Figure 2.





a b

Figure 2: High pressure view cell: flow sheet (a) and photo (b) of the apparatus (60 mL, max. operating pressure 700 bar and max. operating temperature 250℃).

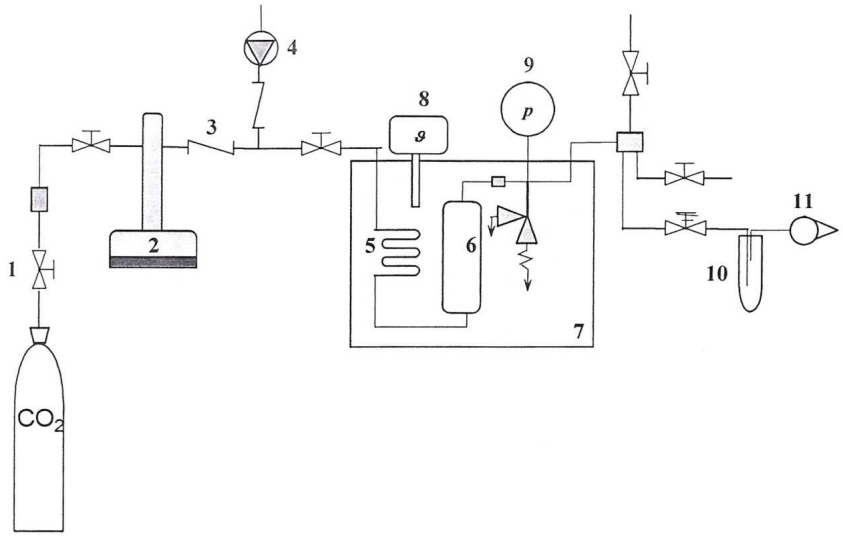
Influence of pressure, temperature and stirring rate on:

- distribution of liquid and gaseous phases – possible phase inversions,
- qualitative evaluation of viscosity of mixtures,
- separation of phases after intensive stirring,

was observed.

High pressure extraction unit

Extraction experiments were performed on extraction unit presented in figure 3. The plant is operating up to pressure 400 bar and temperature 100℃.



1-gas reservoar	5-heater	9-manometer
2-high pressure pump	6-autoclave	10-sampling tramp
3-control valve	7-thermo bath	11-rothameter
4-HPLC pump	8- temperature regulator	V1…V6-valves

Figure 3: High pressure extraction apparatus

RESULTS AND DISCUSSION

Thermodynamic data

The main disadvantage using conventional solvents is that the extracts are contaminated with organic solvents which cannot be easily removed. The legal limitations of solvent residues and solvents (for products for use in human applications) and isolation/fractionation of special components from total extracts in combination with different formulation and sterilization processes will increase the use of dense gases for extraction applications [Stahl et al, 1987].

The solubility of extracted substance in the supercritical solvent is essential for the economy of extraction process. The highest possible loading of SC solvent should be achieved in extraction step of the processes, while in separation step of the process the solubility of solute in solvent should be the lowest [McHugh, Krukonis, 1996].

One of the major advantages of SC fluid extraction processes performed at high pressure - fractionation of extracts decreasing the solvent power of SCF is presented. Active compounds from pumpkin seeds with high contain of oil were very efficiently fractionated [Shi et al, 2007].

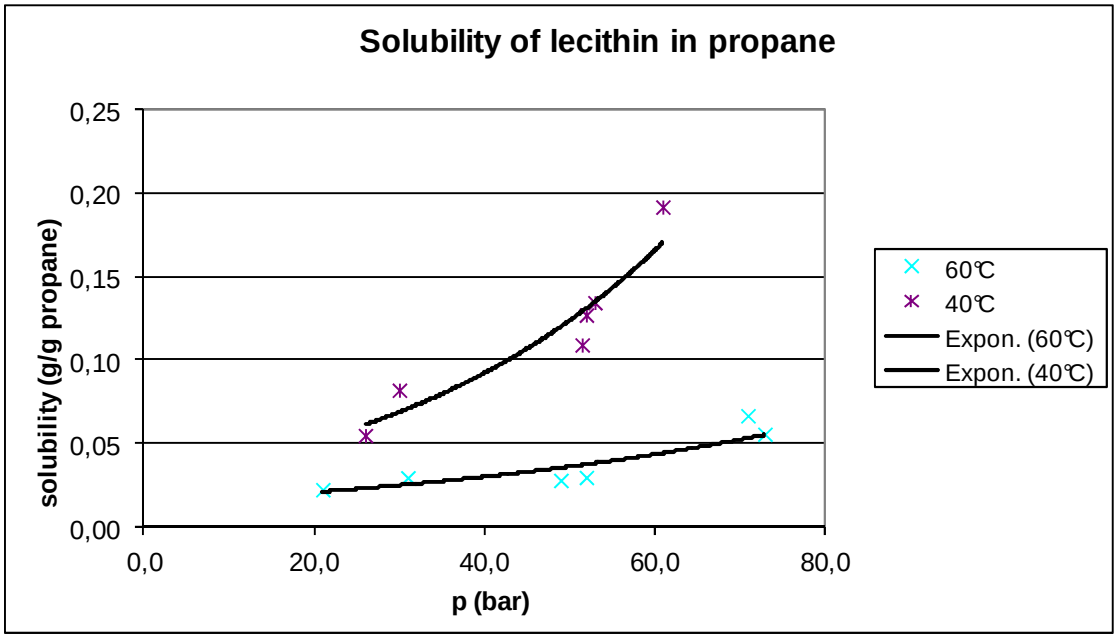


Figure 4: Solubility of lecithin (40%) in propane

Solubility measurements were performed in pressure range up to 70 bar and at temperatures 40℃ and 60℃ and results are presente d in Figure 4.
From the data in Figure 4 it is evident that at temperature 40℃ as well as at temperature 60℃ with increasing pressure from 20 b ar to 60 bar the solubility increases with increasing temperature. The solubility of lecithin in propane at constant pressure decreases with increasing temperature in temperature range from 40℃ to 60℃.

Phase observations for the system lecithin/propane
Phase observations were made at 40℃ and 60℃ in pr essure range from 30 bar to 170 bar. An example is presented in Figure 5. As seen in the figure, the bottom phase is always a lecithin rich phase.

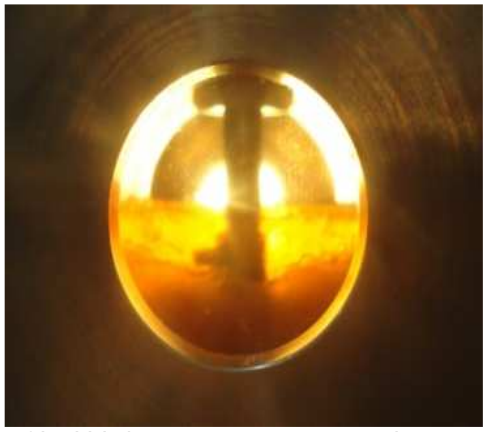


Figure 5**:** Phase behavior of lecithin/propane system at various pressures and temperatures

Extraction experiments

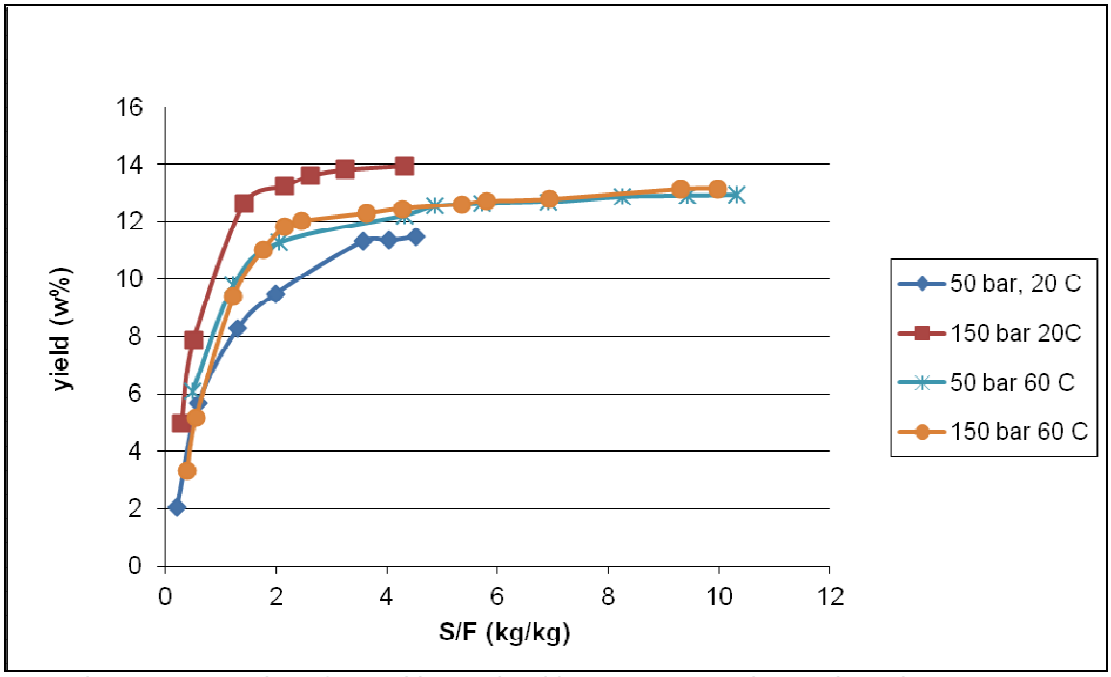


Figure 6: Extraction of pumpkin seeds with propane at 50 bar and 150 bar at 60℃.

At temperature of 20℃ (Figure 6) the yield of extr action is influenced by pressure, at higher pressure yield increases. At temperature of 60℃ the yield is practically independent from pressure.

CONCLUSIONS

Oils from pumpkin seeds, produced by laboratory extraction, were evaluated for phospholipid content. In general, oil had a high level of phospholipids, which was higher in case of SCF extraction at higher temperature and higher pressure.
Separation or formulation of products by supercritical fluids and production of substances and composites with unique properties and characteristics for the use in different applications are nowdays intensively studied. One of the most important advantages of the use of supercritical fluids is selective extraction of components or fractionation of total extracts.
The possible limitation of SCF technologies could be in high investment costs, but the legal restrictions on solvents and solvent residues, fractionation of highly valuable compounds from total extracts in combination with formulation processes will lead to an increase in the use of gasses under high pressure for extraction applications.

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SOME PARAMETERS OF DRIED PORK PRODUCED WITH LOWER SALT CONTENT

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ABSTRACT; Production of meat products with lower salt/sodium content is the goal of today's meat industry because of bad influence of exceed sodium intake by food. In this paper are presented some physico-chemical parameters during processing of dried pork produced with lower salt content. Pork (*m. longissimus dorsi*) was cured with nitrite curing salt in amount of 3 kg/100 kg of meat. In meat were measured the weight loss during curing and drying; moisture content by standard method SRPS ISO 1442:1998, water activity using aw-meter (Wert-Messer, Durotherm) at temperature of 25℃; and pH value by pH-meter (MA-5730; PAT N° 35398, Iskra) according to SRPS ISO 29 17:2004. Average moisture content in dried meat at the end of production was 40.10%. Average weight loss was 2.39% after 7 days of production (after curing) and it is increased up to the end of production, average 34.57%. Acidity of meat during curing, smoking and drying was similar; pH value was around 6.00. Water activity was gradually decreased from average 0.985 after curing (7^{th} day) up to 0.899 at the end of production. During the storage of dried meat under vacuum conditions, pH value decreased from 5.43 in the final product up to 5.11 at the end of storage (120^{th} day). These values are characteristic for curing, drying and fermentation of meat. Dried meat was shelf stable for 120 day under vacuum conditions, without signs of rancidity and without changes in other sensory attributes.
Key words: *dried pork, low salt content, weight loss, water activity, pH value*

INTRODUCTION

Sodium chloride (common salt) is essential ingredient in meat processing which contributes the saltiness (taste), water holding capacity of meat and consequently to textural characteristics (Ruusunen i Poullane, 2005). In modern meat processing, sodium chloride is added mostly through nitrite curing salt. Curing salts with sodium and potassium nitrate are mostly disappeared from industrial meat production, but sometimes they are used in the house-hold manufacturing of dried meat. Salt diffusion in meat and meat dehydration (drying) are common processes but sometimes it is difficult to control them (Arnau et al., 1995).

The first stage in the dry meat processing is dry curing at low temperature, mostly up to 5℃. Curing lasts different time, depending on s hape and size of meat. Curing lasts shortly if meat size is small and at higher temperature; it is also depended on relative humidity (Fantazzinia i dr., 2005). Salt diffusion is key process in dry meat production and solubility of salt on the meat surface is the first factor which regulates salt penetration in meat (Sörheim i Gumpen, 1986; Gil i dr., 1999).

Meat is cured traditionally with 6% of common salt or nitrite curing salt. Present trend in the nutrition is reducing the salt content in meat products, as reported by Ruusunen and Puolanne (2005). According to mentioned the goal of this paper was to investigate some physico-chemical characteristics of dried pork produced with lower salt content, during the curing, drying and storage under vacuum conditions.

MATERIAL AND METHODS

The material in this paper was pork (*m. longissimus dorsi pars thoracis*) originated from white pigs, six months old, with live average weight of 100 kg. After chilling the meat was treated with following mixture: 950 g of nitrite curing salt and 50 g of sucrose, in the amount of 300 g per 10 kg of meat. Curing lasted for 7 days and after that meat was smoked for next 7 days. Process of drying and ripening lasted for 14 days. Final products were packed in PA/PE bags under vacuum conditions and stored at room temperature for 120 days.
Samples for examination were taken on the first day of production (fresh meat), after curing and after 14^{th}, 21^{st} and 28^{th} day of production (final product). In these samples were determined weight loss calculated from differences in the mass of meat during curing, smoking and drying on the scale with sensitivity of 10^{-3}. Moisture content was determined by standard method SRPS ISO 1442:1998; water activity using a_w-meter (Wert-Messer, Durotherm) at temperature of 25℃; and pH value by pH-meter (MA-5730; PAT N°35398, Iskra) according to
SRPS ISO 2917:2004.

RESULTS AND DISCUSSION

The results of weight loss and moisture content of meat are presented in Figure 1. Average moisture content in meat was 77.85%. After curing, moisture content in the superficial layer was 71.59% and in inner part of meat 70.17%. After smoking (14^{th} day of production), moisture content is decreased up to 58.16% in the superficial layer and up to 49.71% in the inner part of meat. During drying of meat (21^{st} day of production), moisture content is also decreased up to 57.20% in the superficial layer of meat and up to 37.72% in the inner part of meat. At the end of production, moisture content was 40.10% in whole dried meat. Consequently to decreasing of moisture content in meat, it was increased weight loss, from 2.39% ± 0.58 in meat after curing up to 34.57% ± 1.48 at the end of production. There are common processes during the dried meat production and according to Incze (1992), the weight loss at meat drying could be very various, from 40 up to 50% and moisture content can be very low (18-22%).

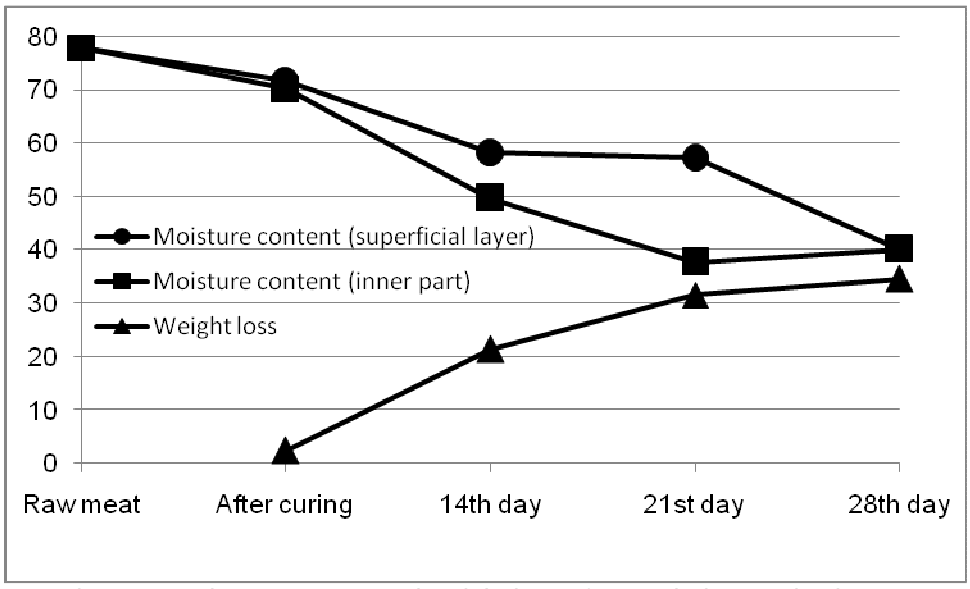

Figure 1. Moisture content and weight loss of meat during production, %

Results of pH value in meat in the different stages of the production are presented in Figure 2.
Value of pH in fresh meat was average 5.83 ± 0.06 and was similar on 7th, 14th and 21st day of production, 5.90 ± 0.09, 5.91 ± 0.07 and 5.86 ± 0.05, respectively. Obtained results for fresh meat are in the accordance with results of Dzierzynska and Pospiech (1989) which cited that pH value of fresh pork is 5.67-5.84 and with the results of Severini et al. (1989) which cited that pH value is 5.48-5.84 in fresh meat after 24 hours of slaughtering.
At the end of production, pH value was decreased up to 5.43 ± 0.01, mostly due to smoking and the influence of organic acid from the smoke. This result is in the accordance with results of Liepe and Porobic (1985) which stated that pH value is 5.30-6.25 in dry hams and with the results of Leon Crespo et al. (1982) that cited pH value of 5.30-5.95 in dry hams. Bellati et al. (1983) cited higher pH value in traditionally produced hams (6.00).
Average pH value in meat after 60 days of storage under vacuum conditions was 5.22 ± 0.05 and after 120 day of storage 5.11 ± 0.05. Lower pH values during the storage under vacuum conditions are the result of activity of lactic acid bacteria that are presented in dried meat in vacuum packaging due to their possibility to survive under microaerophylic and anaerobic conditions. Furthermore, in the curing mixture was added sucrose that was presented source for the growth of these bacteria.

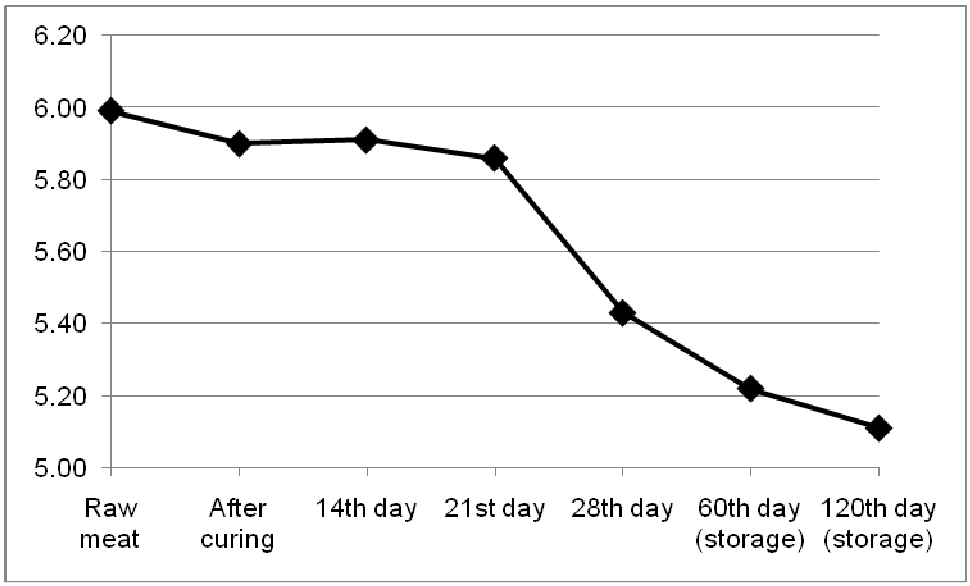

Figure 2. Changes of pH value during production and storage of dried pork

During the curing, smoking and drying, water activity in meat is decreased accordingly with physico-chemical processes (dehydration of meat). Results of the changes of water activity in meat during production are presented in the Figure 3. Average a_w value in fresh meat was 0.992 that decreased after curing (7th day of production) up to 0.985 ± 0.002. After smoking and drying (14th and 21st day of production), a_w value was decreased up to 0.950 ± 0.008 and 0.936 ± 0.009, respectively. At the end of production, a_w value was 0.899 ± 0.004. Obtained results are higher than the results of Leon Crespo et al. (1982) which cited that average a_w value in Jabugo ham of 0.83 (0.75-0.88), but in the accordance with results of Ventanas et al. (1989) which stated a_w value 0.90-0.96 and Molina et al. (1989) which cited that a_w value was 0.83-0.95 in the slow process and 0.87-0.93 in the fast process of the production of dry Iberian ham.

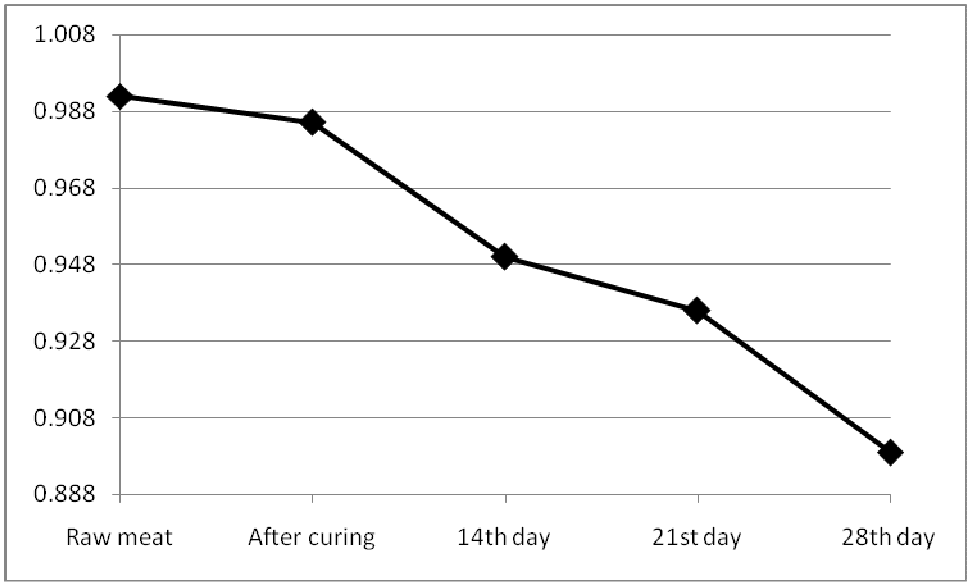

Figure 3. Water activity in dried pork during production

CONCLUSIONS

Moisture content of meat is decreased during the production, while weight loss is increased at the end drying, these values were similar (40.10% and 34.57%, respectively)
Water activity is decreased during drying up to 0.899 at the end of production.
Values of pH were similar during the production (approximately 6.00), but they are lower during the storage of products under vacuum conditions due to the activity of lactic acid bacteria that are presented in the first period of storage.
Lower salt content did not influence mentioned parameters in the comparison with the results of investigation of similar products produced with higher salt content.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 31083 “Reducing of sodium content in meat products - technological posibilities, quality parameters and health aspects (Smanjivanje sadržaja natrijuma u proizvodima od mesa - tehnološke mogućnosti, karakteristike kvaliteta i zdravstveni aspekti)“ financed by the Ministry of Education and Science, Republic of Serbia.

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SUNFLOWER OIL PRODUCTION BYPRODUCT AS A RENEWABLE ENERGY SOURCE

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ABSTRACT: Food and agricultural production often leads to generation of various types of by-products. The same is with sunflower oil production. Sunflower is a crop that is widely grown in Serbia, as well as in the world, mainly for edible oil production, and sunflower husk is a by-product which is generated through this production. It is in accordance with European regulative to find an ecologically friendly way of disposal for this kind of waste. Sunflower husk falls in the category of biomass and can be used for energy generation. The most common way to utilize biomass for energy generation is by combustion. In this study sunflower husk was investigated as an energy source, and it was pelleted in order to achieve better energy conversion and combustion characteristics. The optimal conditions of pelleting process were determined, that is optimal water content in the material for pelleting. When the material is well prepared less energy is consumed during pelleting process.
The aim was to analyze energy and combustion properties, as well as physical and chemical properties of pellets made from sunflower husk. Sunflower husk had satisfactory characteristic as a biomass fuel, although its pelleting required a lot of energy, and the pellets had poor abrasion characteristics (around 7-14%). The heating value was around 19.00 MJ/kg, and the volatile matter just above 70%. These values are mostly in accordance with European fuel pellets standards, with some aberrations for some of the properties. Since these standards are manly for wood, and wood-residue pellets, lower criteria could be applied for agricultural biomass pellets. Basic chemical and ultimate analyses of sunflower husk were in accordance with literature data for biomass samples, and low level of sulphur were detected (< 1.00%).
Key words: *sunflower husk, pelleting, fuel pellets, biomass*

INTRODUCTION

In food and agricultural production a substantial amounts of residues are generated, which are not exploited enough and are often treated as waste (Demirbas et al., 2009). They present valuable biomass and could be used as a renewable energy source, which presents an ecologically friendly way of utilization. These residues often have an unfavourable shape, therefore are often densified in pellets, which are easier to manage and transport. Most common way of generating energy from biomass is direct combustion, where pelleted material also presents benefits as pellets have more complete combustion and are easier to feed into the combustion chamber, in comparison to bulk material. The European biofuel pellet market is increasing over the last couple of years (Di Giacomo and Taglieri, 2009; Samuelsson et all., 2009), as pellets are becoming more popular for heating.
In order to produce quality pellets the material needs to be properly prepared in terms of granulation and moisture content (Kaliyan and Morey, 2009), therefore sometimes it is necessary to mill and condition the material. Conditioning is a process where material is exposed to water steam on elevated temperature and pressure, and thus becomes more suitable for densifiction. Too much water or too high temperatures can have a negative effect on production capacity and specific

energy consumption in the pelleting process, as well as on pellet quality (Thomas et al., 1997; Sredanović and Lević, 2000). Therefore it is necessary to determine the optimal pelleting parameters for every material.
Sunflower cultivation is taking up around 160 000 ha in Serbia, while around 90% of that production is in Vojvodina (Knežević and Popović, 2011). After the production of sunflower oil, a large quantity of husk remains, which could be used as biofuel. In order to do so the material should be analysed, and prepared (pelleted).
In this study four ways of material conditioning were investigated, and obtained pellets were analyzed for basic quality parameters. The sunflower husk was also analyzed to determine combustion properties and suitability of this material for biofuel.

MATERIAL AND METHODS

Material and processing

The sunflower husk was obtained from oil factory Victoria Oil, Šid, Serbia, in 2010, and all technological operation processing was performed at pilot-plant facility of Institute of food technology in Novi Sad, Serbia.
In order to achieve better granulation and more efficient pelleting process, material was milled using hammer mill (ABC inženjering, Serbia) equipped with sieve with 4 mm openings. To determine particle size distribution of milled material standard sieving was performed using sieves with following diameter of openings: 2500, 2000, 1250, 1000, 800, 630, 250, 125 and 63 μm.
Material was divided in four batches of 25 kg each and conditioned using a double-shaft pedal mixer/steam conditioner (Muyang SLHSJ0.2A, China). Two of the batches were conditioned by combined application of steam and water, with steam pressure of 2 bars, which was added to the material until it reached temperature of 80℃. Next two batches were conditioned only by add ition of water.
Pelleting was conducted on a flat die pellet press 14-175, AMANDUS KAHL GmbH & Co. KG (Germany), with die openings diameter of 6 mm and die thickness 30 mm. After pelleting, pellets were cooled and stored for 24 hours under room conditions, to reach equilibrium moisture.

Physical analysis

Abrasion of pellets was determined by “Pfost” abrasion test unit, Bühler, Switzerland (Pfost and Allen, 1962; Pfost, 1963). The test unit is a tumbling can device, where abrasion is induced by pellets shearing over each other and over the wall of drums, producing fines. The dimensions of the drum are specified, and a sample of 500 g of sieved pellets is inserted inside it. The device tumbles the pellets for 10 min at 50 rpm (rounds per minute), after what pellets are sieved through a sieve with a grid size of 0.8 x pellet diameter. The amount of fines passing through the sieve was determined and expressed in percentage.
Bulk density of grounded and pelletized material was measured with a bulk density tester, Tonindustrie, West und Goslar, Germany.

Chemical analysis

Starting material was analyzed for moisture content (NREL/TP-510-42621), crude ash (NREL/TP-510-42622), crude fat (AOAC 920.39), crude fibre (AOAC 962.10). In pellets, beside moisture and ash, were also analyzed volatile mater (CEN/TS 15148:2005), fixed carbon (calculated by difference between 100 and the sum of volatile matter, ash and moisture), content of C, H, N (CEN/TS 15104:2005) and S (CEN/TS 15289:2006) and higher heating value (HHV) (CEN/TS 14918:2005).

Statistical analysis

Statistical Analysis System (Statistical, Tulsa, Oklahoma, USA) was used for analyzing variations (analysis of variance – ANOVA) and least significant differences (LSD). The level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Starting material

The analysis of the starting material is presented in table 1. The majority of chemical composition belongs to crude fibre, while the contents of protein and fat are around 4%. This content of fat was proved to be helpful in the process of pelleting.

Table 1. Chemical composition of milled sunflower husk

Component	Concentration (% (w/w))
Moisture	8.63
Crude ash	3.24
Crude protein	4.15
Crude fat	4.25
Crude fibre	52.53

After milling, geometric mean particle size of the material was 888.2 μm and geometric standard deviation was 1.84. Milling of the material is beneficial as decreasing particle size enables better absorption of water steam during conditioning. Smaller particle size also improves pellet quality by reducing air spaces between particles and weak spots in the pellets (Kaliyan and Morey, 2009).

In the process of conditioning, water can be added to the material in a form of liquid or steam. As steam conditioning requires more energy consumption, sometimes it can be replaced with liquid water conditioning. Energy is saved as water is added at room temperature, and it doesn't require heating and achieving elevated pressure. In this experiment both methods of water addition were investigated.

Batches conditioned by combined application of steam and water had final moisture content of 26.70% (s-1), and 23.25% (s-2) respectively. The moisture in s-2 batch was lowered in order to obtain pellets with satisfying quality and less energy consumption for drying of product. In the third (w-1) and fourth batch (w-2) that were conditioned only by water addition, water was added in order to reach similar moisture content to first and second batch, and achieved moisture contents were 26.05% (w-1) and 22.76% respectively (w-1).

Abrasion of pellets is shown in figure 1, where it is noticeable that each material had different abrasion. In general steam conditioning raises moisture content of the material, but at the same time it exposes it to elevated temperatures, which facilitates particle binding (Thomas et al., 1997). On the other hand water conditioned pellets in this case had the lowest abrasion, when a lower amount of water was applied (w-2). The highest abrasion was determined for water conditioned pellets with higher moisture content applied (w-1). Samuelson et al (2009) concluded that water has significant influence on particle binding in pellets, which results with influence on abrasion. They investigated wood pellets and found that higher moisture content (around 11,5%) resulted in lower abrasion (1-2%). But increasing moisture content can have a positive effect only to a certain point, while excessive moisture can cause the opposite effect (Fasina 2008; Kaliyan and Morey, 2009). Therefore it is necessary to discover the optimal moisture content for each raw material, depending on its

chemical composition and physical properties. When fuel pellets are considered, higher abrasion can cause malfunctioning of transporting and feeding systems, and causes larger dust emissions (Van Loo and Koppejan, 2008). All of the samples had significantly higher abrasion than proscribed by EU standards (< 2.3% in ÖNORM M 7135, and DIN 51731)

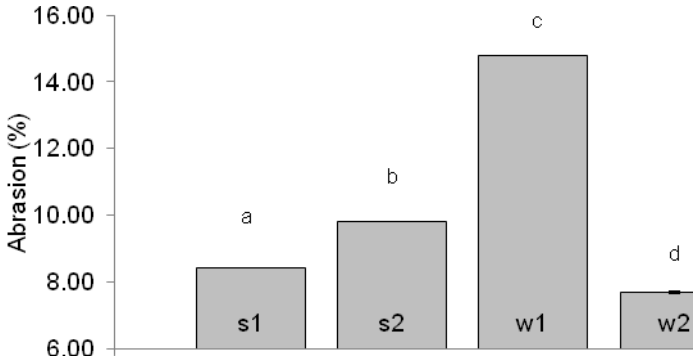

Figure 1. Abrasion of pellets

[abcd]Means with different letters in the same row are significantly different at the 5 % level

Bulk density was the highest for w-2 samples (figure 2), which were conditioned with water, and had final moisture of material 22.76%. The lowest density was measured for w-1 samples. In general all of the samples had poor bulk densities, comparing to other biomass samples (Samuelson et al, 2009; Mirnada et al, 2011; Zamorano et al, 2011), which have bulk densities above 500 kg/m^3.

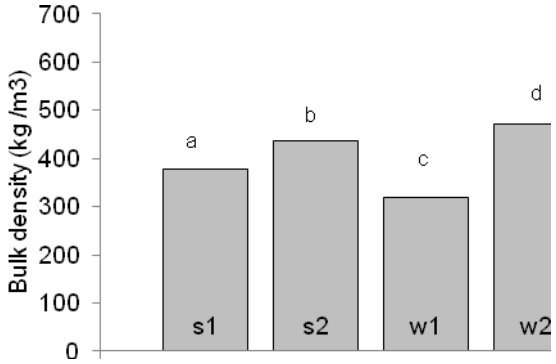

Figure 2. Bulk density of pellets

[abcd]Means with different letters in the same row are significantly different at the 5 % level

Chemical analyses of pellets are shown in table 2. Moisture content of the pellets was below 10%, as proscribed by ÖNORM M 7135 and DIN 51731, while ash content was around 3%, which is higher than proscribed, but in accordance with values typical for agricultural and food residues. Volatile mater and fixed carbon values are also in accordance with literature data (Zabaniotou et al, 2008; Khan et al, 2009; Raclavska et al, 2011). Ultimate analyses were characteristical for biomass, with emphases on sulphur and nitrogen levels which were low, and that is important for off gas emissions, when pellets are combusted. Heating values were around 19 MJ/kg, and proscribed values for fuel pellets are >18 MJ/kg (ÖNORM M 7135, DIN 51731).

Table 2. Results of pellet analyses

	0	S-1	S-2	w-1	w-2
PROXIMATE ANALYSES					
Moisture (%)*	8.63±0.21	8.78±0.04[a]	8.59±0.06[a]	9.94±0.18[b]	8.84±0.33[a]
Ash (% db)	3.24±0.06	3.29±0.02[a]	3.27±0.03[a]	3.26±0.02[a]	3.24±0.05[a]
Fixed carbon (% db)	18.86±0.06	16.31±0.61[a]	16.29±0.46[a]	15.56±0.28[a]	15.86±0.28[a]
Volatile matter (% db)	69.27±0.06	71.62±0.61[a]	71.85±0.46[a]	72.06±0.28[a]	72.06±0.28[a]
ULTIMATE ANALYSIS					
C (% db)	46.24±0.69	46.49±1.03[a]	45.90±0.72[a]	45.82±1.02[a]	46.05±0.73[a]
H (% db)	6.18±0.18	6.06±0.08[a]	5.74±0.23[a]	5.90±0.29[a]	5.88±0.35[a]
N (% db)	0.66±0.03	0.66±0.04[a]	0.65±0.06[a]	0.62±0.03[b]	0.61±0.04[b]
S (% db)	0.07±0.01	0.06±0.01[a]	0.06±0.01[a]	0.06±0.01[a]	0.06±0.01[a]
HHV (MJ/kg, db)	18.85±0.05	19.11±0.29 [a]	19.23±0.02 [a]	19.23±0.04 [a]	19.18±0.01 [a]

[abcd]Means with different letters in the same row are significantly different at the 5 % level
*determined after 24 hours

CONCLUSIONS

Sunflower husk pellets had satisfactory characteristics, and high heating value, although their physical properties were somewhat poor. The results showed that steam conditioning can be replaced with water conditioning, which brings energy savings to the whole process. The pellets for which the material was conditioned with lower moisture content, proved to have the best physical characteristics.

ACKNOWLEDGEMENTS

This study was financed by the Ministry of Education and Science, Republic of Serbia, project III 46005.

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THE EFFECT of SPRAY DRYING PROCESSING CONDITIONS on PHYSICAL PROPERTIES of SPRAY DRIED MALTODEXTRIN

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ABSTRACT: Maltodextrin was subjected to spray drying to determine the effect of spray drying conditions on moisture content, water activity, particle properties (particle size distribution and particle density) and bulk properties (bulk and tapped densities, porosity, flowability) of the powder product. Experiments have been performed in a pilot scale spray-dryer (Mobile Minor Niro-Atomizer, Denmark) using a full-factorial design to provide data and correlations that predict the powder properties as a function of the main operational variables of the spray-dryer. The inlet (170-190℃) and outlet air temperatures (60-90℃) and the atomization pressure (196-392 kPa) were investigated as spray drying process variables. The effect of spray drying conditions on physical properties of powders was expressed with perturbation graphs. Perturbation graphs revealed that outlet air temperature and atomization pressure had more effect than inlet air temperature, on the physical properties of maltodextrin powder. The results showed that the Sauter mean diameter ($D_{3,2}$) was between 6.728 and 12.87 µm for maltodextrin powders . The bulk densities of samples were changed in the range of 476 and 568 kg/m^3. Maltodextrin powders had bad flowability due to their small particle size.

Key words: *Maltodextrin, spray drying, particle and bulk properties, particle size distribution*

INTRODUCTION

Spray drying is a well-established and widely used method for transforming a wide range of liquid food products into powder form. The process involves spraying finely atomized solutions into a chamber where hot and dry air rapidly evaporates the solution leaving the spray-dried particles. Spray-dried powders can be stored at ambient temperature for prolonged periods without compromising the powder stability. They are also cheaper to transport and easier to handle in manufacturing plants (Koç et al. 2010; Jayasundera et al., 2011; Koç et al. 2011).

However, caking or stickiness as one of the major degradation problems hindered the development of powders (Adhikari et al., 2007). The problem is mainly due to the existence of low molecular weight sugars with low glass transition temperatures. To produce food powders, using maltodextrins as a drying carrier is a popular method nowadays (Bhandari et al., 1997; Ersus and Yurdagel, 2007; Wang and Zhou, 2012). Maltodextrin can significantly increase the glass transition temperature and reduce the hygroscopicity of dried products (Goula and Adamopoulos, 2010).

Maltodextrin, a common encapsulating material used in the food industry, is made by the hydrolysis of starch, and comes in the form of a white powder and has a sweet taste. However, there have been very limited researches on the physical properties of maltodextrin, which can affect the resultant powder quality.

Physical properties of food powders including the particle shape, density and porosity, surface characteristics, diameter, and size (Kurozawa et al., 2009) can be affected by the spray drying temperatures and the type of atomizer, that are important in the storage, handling and final application of powder product (e.g. particle and bulk properties). One of the most important physical properties of

powders with regards to handling is particle size. Particle size can influence flow out of storage bins, the blending of different components, compaction, and the segregation of a mixture, in which smaller particles stay distributed on the bottom and larger particles on top. In addition, these properties significantly influence the essential properties of food products such as smell, texture, and appearance. The knowledge of food density is of fundamental use for material properties studies and for industrial processes in adjusting storage, processing, packaging, and distribution conditions. Bulk density includes the volumes of the solid and liquid materials and all pores and is generally used to characterize a final product obtained by drying (Kurozawa et al. 2009; Koç et al., 2012).
In this study, it was aimed to determine the influence of spray drying process, in terms of inlet and outlet air temperatures and the atomization pressure, on moisture content, water activity, particle properties (particle size distribution and particle density) and bulk properties (bulk and tapped densities, porosity, flowability) of maltodextrin.

MATERIAL AND METHODS

Maltodextrin (DE=18), used as the test material was supplied from Çağdaş Kimya, Turkey. Maltodextrin was dissolved in the distilled water and the solution containing 40% maltodextrin was used in the experiments.

Spray Drying

Experiments were conducted in a pilot scale spray dryer (Mobile Minor Niro-Atomizer, Denmark). Maltodextrin solution was atomized from nozzle into vertical, co-current drying chamber, 0.87m diameter and 1.2m height, under various operating conditions. Feed temperature of below 10℃ and hot air flow rate of 1.54 m^3/min were fixed for all experiments. Air inlet temperature (170-190℃), air outlet temperature (60-90℃) and atomization pressure (196-392 kPa) we re adjusted according to the full-factorial experimental design (Table 1). Air outlet temperature was controlled by regulating the feeding velocity. The dried powder was collected in a single cyclone separator and then packaged in ALPE packaging material until used for analysis.

Moisture content

Moisture content of maltodextrin powders was measured with a halogen moisture analyzer (Ohaus MB45, Switzerland) at 105℃.

Water activity (a_w)

The water activity (a_w) values of powders were measured with a water activity measurement device (Testo AG 400, Germany), with a ±0.001 sensitivity.

Particle Properties

Particle size distribution: The particle size distribution of the maltodextrin powders were measured using a laser light diffraction particle size analyser (MasterSizer model S 2000, Malvern Instruments Ltd., Worcestershire, U.K.) in which a small quantity of the powder was dispersed in water and the particle distribution was monitored during five successive trials. The particle size was expressed as mean volumetric size $D_{3,2}$ (Sauter mean diameter), and was calculated as follows:

$$D_{3,2} = \frac{\sum_{1}^{i} D_i^3 f_i}{\sum_{1}^{i} D_i^2 f_i} \quad (1)$$

where f_i is the frequency of particles of diameter D_i.

Particle density: Particle density (ρ_p) of the powder samples was analysed according to a study by Barbosa-Cánovas et al. (2005). The liquid (petroleum ether) pycnometry can be used to determine particle density depending on the volume of pycnometer bottle used.

Bulk Properties

Bulk and tapped densities: The bulk density (ρ_b) of the powders was determined by measuring the weight of the powder and the corresponding volume. Approximately 20 g of powder sample was placed in a 100 ml graduated cylinder. The bulk density was calculated by dividing the mass of the powder by the volume occupied in the cylinder. For the tapped density (ρ_t), the cylinder was tapped vigorously by hand until no further change in volume occurred (Jinapong et al., 2008).

Porosity: Porosity (ε) of the powder samples was calculated using the relationship between the tapped (ρ_t) and particle (ρ_p) densities of the powders as shown below (Jinapong et al., 2008):

$$\varepsilon = \frac{(\rho_p - \rho_t)}{\rho_t} \times 100 \quad (2)$$

Flowability: Flowability of the powders was evaluated in terms of Carr index (CI) (Carr, 1965) CI was calculated from the bulk (ρ_b) and tapped (ρ_t) densities of the powder as shown below:

$$CI = \frac{(\rho_t - \rho_b)}{\rho_t} \times 100 \quad (3)$$

Statistical Analysis

All samples were analyzed in triplicate. The analysis of variance (ANOVA) at a confidence level of 95% was performed. All the results that were obtained were analyzed using Design Expert–version 7.0 software (Statease Inc., MI, USA).

RESULTS AND DISCUSSION

Results of 11 different experiments (different runs of spray drying) performed according to full-factorial design were also given in Table 1.

Maximum moisture content (5.33%, wb) and water activity (0.216) values were recorded at the same spray drying conditions; outlet air temperature of 70℃, inlet air temperature of 190℃ and atomization pressure 392 k Pa. Moisture content and water activity of the maltodextrin powder increased with a decrease in the outlet air temperature since the feed flow rate to atomizer and also spray dryer is high to achieve lower outlet air temperatures, in which case the moisture removal rate gets lower. According to perturbation graphs (Figure 1a and 1b) moisture content and water activity were affected significantly by the outlet air temperature.

Table 1. Full Factorial Design with experimental values of response variables

T_{in} (℃)	T_{out} (℃)	P (kPa)	Moisture Content % (wet basis)	a_w	$D_{3,2}$ (μm)	ρ_b (kg/m^3)	ρ_t (kg/m^3)	ρ_p (kg/m^3)	ε (%)	CI (%)
170	70	196	4,430	0,160	10,28	549	649	1185	82,63	15,39
190	70	196	4,730	0,194	9,374	567	780	1351	73,29	27,35
170	70	392	5,120	0,194	9,328	554	732	1266	72,90	24,33
190	70	392	5,330	0,216	6,728	485	809	1306	61,37	40,06
180	80	294	3,540	0,128	9,947	568	748	1253	67,60	24,09

180	80	294	4,740	0,203	9,915	540	780	1292	65,66	30,84
180	80	294	4,480	0,119	9,931	548	714	1174	64,43	23,31
170	90	196	3,030	0,111	12,87	544	765	1063	38,99	28,80
190	90	196	3,330	0,115	11,02	490	788	1189	50,85	37,76
170	90	392	3,500	0,127	8,913	476	673	1173	74,31	29,24
190	90	392	2,880	0,107	9,502	504	695	1133	63,00	27,52

The particle size distribution of spray dried maltodextrin powders was shown in Figure 2. All powder particles had a narrower particle size range with relatively uniform distribution. The mean particle size of a material may greatly influence its reactivity and the quality of the end product (Baranauskiene et al., 2006). The results showed that spray drying does not produce larger particles. The Sauter mean diameter ($D_{3,2}$) of powders ranged from 6.728 to 12.87 µm. Perturbation plots (Figure 1c) showed that the Sauter mean diameter ($D_{3,2}$) of the maltodextrin powder significantly affected all the independent variables (outlet and inlet air temperatures and atomization pressure). Perturbation graphs revealed that outlet air temperature and atomization pressure had more effect than inlet air temperature, on the Sauter mean diameter ($D_{3,2}$).

The bulk properties (bulk and tapped densities, porosity, flowability) of a food powder are highly dependent on particle size and its distribution (Barbosa-Cánovas et al. 2005). Lower bulk densities of a product are not desirable, resulting in a greater volume of package. Moreover, lower the bulk density, more occluded air within the powders would be and a greater possibility for product oxidation resulting in reduced storage stability (Goula and Adamopoulos 2008; Kurozawa et al. 2009). The bulk densities of samples were changed in the range of 476 and 568 kg/m^3 (Table 1). Bulk density was affected by all the independent variables (Figure 1d) whereas tapped density and particle density was affected by only inlet air temperature and outlet air temperature, respectively (Figure 1e and 1f).

Minimum flowobility value (15.39%) and maximum porosity value (%82.63) were recorded at the same spray drying conditions; outlet air temperature of 70 ℃, inlet air temperature of 170℃ and atomization pressure 196 k Pa. Spherical particles pack the best and thus, have the highest bulk densities and porosity (Reineccius 2004). Maltodextrin powders have moderate-poor flowability due to their small particle size. Porosity was influenced by the outlet air temperature and atomization pressure. However, flowability was influenced by only inlet air temperature (Figure 1g and 1h).

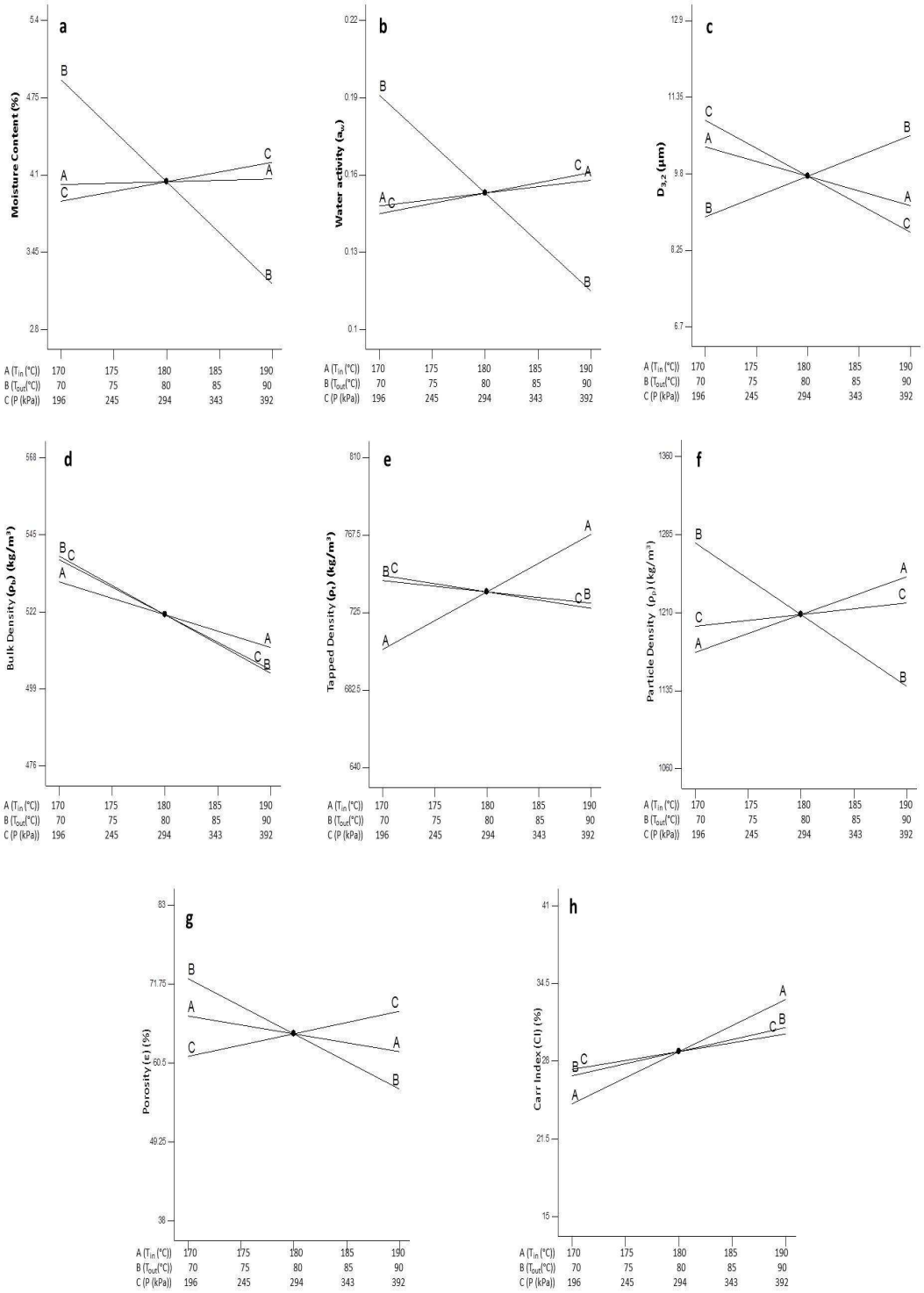

Figure 2. Perturbation plots. (A) Inlet air temperature, (B) Outlet air temperature, (C) Atomization pressure

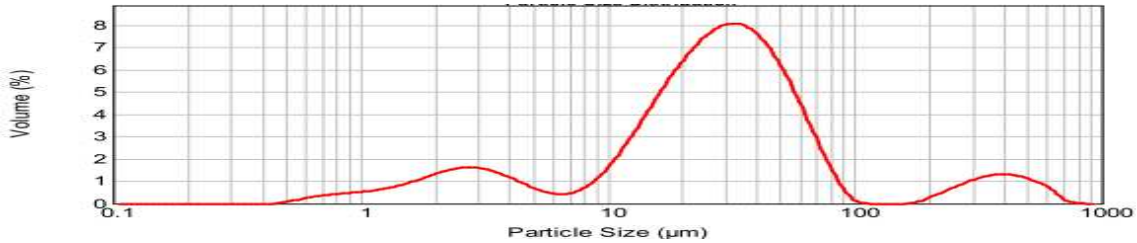

Figure 3. The particle size distribution of spray dried maltodextrin (inlet air temperature of 170℃, outlet air temperature of 70℃ and atomizati on pressure of 392 kPa)

CONCLUSIONS

In this study, physical properties of maltodextrin powders were investigated. The results showed that spray drying does not produce larger particles. Perturbation graphs revealed that outlet air temperature and atomization pressure had more effect than inlet air temperature, on the physical properties of maltodextrin powder. Maltodextrin powders had bad flowability due to their small particle size.

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USING THE IMPACT DETACHERS AT THE HEAD OF THE REDUCTION SYSTEM IN THE WHEAT FLOUR MILLING PROCESS

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ABSTRACT: In flour mills impact detachers are most commonly applied to support the grinding of clean middlings (clean endosperm with minimal bran contamination) at the head of the reduction system supplementing the roller mill before sifting. However, using the impact mills, the conditions for controlled milling are less favorable and could result in deterioration of the flour quality. The purpose of this work was to examine the effect that impact detachers have on the milling results on the front passages of the reduction system. The streams entering and leaving the impact detachers applied on the different reduction passages (1M, 2M and 3M) in the industrial mill were intercepted and employed in the experiments. The changes in the particle size distribution and ash content of the material were followed. Results show that impact detachers significantly increase the degree of particle size reduction of the feed. Under dominant impact forces both endosperm and bran particles are fractured limiting their use to the passages with the low ash content of the stock (<0,6%). Otherwise, the fractured bran particles would pass into the flour causing the deterioration of the flour quality.
Key words: *flour milling, reduction system, impact detacher, milling results*

INTRODUCTION

Wheat flour milling uses repeated breakage of the stocks and separation (by sifting) to achieve effective removal of bran and germ from endosperm, and appropriate size reduction of the endosperm to produce flour relatively free from bran contamination (Campbell and Webb, 2001; Sudgen and Osborne, 2001). During any comminution operation, both material properties and the design and operation of the milling equipment affect particle breakage [Scanlon and Lamb, 1995; Campbell et al., 2001). In flour milling, the predominant comminution tool is the roller mill (Haque, 1991) in which the feed material is passed between two counter-rotating rolls of usually 250 mm diameter and with either a corrugated or smooth finish. The rolls are separated by a small gap and rotate at different speeds. In a roller mill, particles are subjected to shear and compressive forces. Particle size reduction is achieved by passing cleaned and conditioned wheat through a series of break (fluted) and reduction (smooth) rolls (Posner and Hibbs, 2005).

The main goal of the flour milling industry is to produce a selection of flour of defined quality followed by low investment and energy costs (Fistes et. Al, 2008). For this reason flour mills are forced to rationalize their production facilities by reducing the equipment, operating and maintenance costs (Baltensperger, 1993; 2001). The trend in recent years has been to shorter mill flows, necessitating harsher grinding on both break passages and reduction passages which could have significant consequences to milling results (Dexter, 1996). Also, the grinding has been supported with impact milling of separate or together with roll passes making the shorter roll surface a reality (Wanzenried, 1991).

The main characteristic of the impact type of mills is the use of impact as the principal size-reducing force (Haque, 1991). The material is reduced by the loss of kinetic energy when the particle is struck by a high-velocity impeller or accelerated

and thrown against a wall to stress the particle beyond its elastic limits (Posner and Hibbs, 2005). Different types of impact machines are used in flour mills and, depending on their use, they are designated as impact detachers, sterilizers and entoleters. They are used: on wheat to break infested kernels and destroy insects and insect eggs, to support the grinding by supplementing the roller mills, or even to completely replace the rolls in both break and reduction systems in order to increase capacity and flour extraction. Most commonly, impact detachers are applied to support the grinding of clean middlings (clean endosperm with minimal bran contamination) at the head of the reduction system. These machines usually follow the rolls ahead of the plan sifter (Posner and Hibbs, 2005). The use of this machines increases flour yields and improves the sifting efficiency. It also prevents stock from over-tailing these sieves and loosing good quality stock to by-products.
However, under predominant impact forces both endosperm and bran particles are fractured making the conditions for controlled milling less favorable and could result in deterioration of the flour quality. The purpose of this work was to examine the effect that impact detachers have on the milling results on the front passages of the reduction system. The changes in the particle size distribution and ash content of the material were followed.

MATERIAL AND METHODS

The streams entering and leaving the impact detachers applied on the different reduction passages (1M, 2M and 3M) in the industrial mill were intercepted and employed in the experiments. Before sampling, the mill was checked for any kind of disturbances in the process and sampling was carried out with balanced load to machines. Since the impact detachers are used diagrammatically between the roller mill and the plan sifter the samples of the stock leaving the impact mills were taken after the air locks of the pneumatic conveying system before entering the appropriate section of the sifter. The mass of the each sample was around 2 kg.
Three set of batches of 100 g of the each sample (stocks entering and leaving the impact detachers applied on the mentioned passages) were sieved for 3 min on a Bühler laboratory sifter (gyratory in a horizontal plane), model MLU-300 (Uzwil, Switzerland). The sieve openings were 500, 350, 250 and 150 μm, and a bottom collecting pan was fitted.
Moisture and ash contents of the samples were determined according to ICC standard methods no. 110/1 and 104/1, respectively.
The significance of the differences in flour yield and flour ash content between the stocks that enter and leave the impact mills have been tested by the paired Student's *t*-test.

RESULTS AND DISCUSSION

Results show that impact detachers significantly increase the degree of particle size reduction of the feed (Table 1).

Table 1. Particle size distribution of the stocks entering (E) and leaving (L) the impact detachers

passage	stock	size fraction yield (%)				
		>500 μm	**500/350 μm**	**350/250 μm**	**250/150 μm**	**<150 μm**
1M	E	0,2	0,5	21,0	38,8	39,5
	L	0,1	0,3	9,5	25,9	64,2
2M	E	1,2	9,2	41,6	28,9	19,1
	L	0,5	3,6	28,2	28,7	39,0
3M	E	0,4	3,2	14,8	12,1	69,5
	L	0,4	1,1	11,2	8,1	79,2

The quantity of flour (<150 □m) increased while the quantity of the coarse (>500 and 350-500 □m) and especially medium sized stocks (150-350 □m) decreased, especially on the 1M and 2M. The difference in flour yield is statistically significant ($p<0.001$). The results confirm the role of the impact detacher in wheat flour milling process as a support to the roller mills for additional grinding of the stock before sifting.

Using the impact detachers the ash content of both the flour (<150 □m) and the rest of the stock (>250 □m) increases (Table 2). As it was mentioned earlier in paper, the nature of deformation during comminution operation depends not only on the applied stresses but as well on the particle components upon which the stresses act. The middlings are composed primarily of endosperm, but they also contain adhering bran and germ. Ash is concentrated in the bran and the ash content increases from the inner to the outer part of the wheat kernel (Pomeranz, 1988). Considering the present grinding conditions, under dominant impact forces both endosperm (brittle material) and bran particles (tough and fibrous) are fractured and the flour ash content increases. This increase in flour ash content follow the increase of the ash content of the feed material sent to the impact detachers, being 0,53, 0,76 and 1,03 $(\%)_{dm}$ for 1M, 2M and 3M respectively. On 1M and 2M, the difference in ash content of the flour before and after the impact detachers at the 0,05 level is not statistically significant but at the 0,01 level is statistically significant. On 3M this difference is statistically significant on both 0,05 and 0,01 level.

From the technological point of view, the increase of the flour ash content on 2M is already high while on 3M is not acceptable. Practically the use of impact detachers is limited to the passages with the low ash content of the stock (<0,6%) that is clean middlings with minimal bran contamination. Otherwise, the fractured bran particles would pass into the flour causing the deterioration of the flour quality.

Table 2. Moisture and ash content in the size fraction of the stocks entering (E) and leaving (L) the impact detachers

passage	stock	moisture content (%)		ash content $(\%)_{dm}$	
		>250 □m	<150 □m	>250 □m	<150 □m
1M	E	11,9	13,3	1,18	0,37
	L	11,3	13,2	1,44	0,42
2M	E	12,6	13,2	1,16	0,40
	L	11,8	13,0	1,55	0,48
3M	E	10,3	12,4	3,48	0,48
	L	10,0	12,0	3,92	0,61

The results also show a relatively small decrease in moisture content in the size fractions of the stock following the use of impact detachers (Table 2). However, it is expected considering that sampling was carried out after the air locks of the pneumatic conveying system and that the stock was in contact with the air from the pneumatic conveying system. In addition to that, impact detachers significantly increase the degree of particle size reduction of the stock and therefore its specific surface area practically increasing the drying effect of the pneumatic air.

CONCLUSIONS

Impact detachers significantly increase the degree of particle size reduction of the feed. Under intensive impact forces, as a predominant stress causing the comminution, both endosperm and bran particles are fractured. The fractured bran particles could pass into the flour and cause the deterioration of the flour quality (as determined by ash content). Considering that the aim of the wheat flour milling process, along with the size reduction, is to obtain the best possible dissociation of the starchy endosperm from the other parts of the grain, the application of impact

detachers as a grinding support to roller mills needs to be limited to the passages with the low ash content of the stock (<0,6%).

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR031014 “Razvoj novih funkcionalnih konditorskih proizvoda na bazi uljarica (Development of the new functional confectionery products based on oil crops)”, financed by the Ministry of Science and Technological Development, Republic of Serbia.

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CHANGES IN THE QUALITY OF WHEAT FLOUR AS A RESULT OF USING INERT DUSTS IN ORGANIC SYSTEMS OF PROTECTION AGAINST STORAGE INSECTS

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ABSTRACT: The aim of the work was to describe the impact of inert dusts as agents officially approved for use in the storage of crops from certified organic production system on the technological quality of wheat flour. In order to control storage insect pest *Sitophilus oryzae* (L.*)* in wheat, various inert dusts were used: natural zeolite, two products of diatomaceous earths originating from Serbia and a commercial **product** Protect-It®. Wheat samples, infested and non-infested, treated with the inert dusts were milled and the obtained flour samples were analyzed on rheological behaviour using Mixolab®. The nine parameters obtained were analyzed using the method of Principal Component Analysis (PCA). It was found that the two principal compounds accounted for 75.6% of total variance in the data. This research showed that dough stability (0.96) and water absorption (0.90) contributed most to the first principal component, starch gelatinization (0.83) and stability of the hot-formatted gel to the second one. Flour obtained from wheat treated with inert dusts showed higher water absorption and stability during mixing, and less protein weakening during heating in comparison to the control wheat non-treated with inert dusts. The component scores indicated the similarities among the treatments with inert dust. The outlier sample was the control with insect infestation and without inert dust protection.
Key words: *insect infested and non-infested wheat, inert dusts, Mixolab®, PCA*

INTRODUCTION

Natural zeolite (NZ) and diatomaceous earth (DE), commonly designated as inert dusts, are in some cases alternatives for conventional insecticides and fumigants. Codex Alimentarius Commission (1999) recommends control of insect pests in food commodities and lists zeolite and diatomaceous earth as permitted substances in organic food production and in plant pest control.

DE is a naturally occurring siliceous mineral compound from marine sediments which consists of microscopic skeletal remains of single-celled algae (phytoplankton) called diatoms. It is composed of amorphous silicon dioxide that is non-toxic to mammals (Korunić et al., 1998).

There are several research works dealing with the effectiveness of DE (Korunić et al., 1998; Fields and Korunić, 2000; Vardeman et al., 2007) and NZ (Kljajić et al., 2010) against stored-grain insects. Furthermore, few authors addressed the effects inert dusts treatments exert on the technological quality of grain and flour (Desmarchelier and Dines, 1987; Korunić et al. 1996).

The Mixolab® instrument measures the behaviour of wheat protein and starch when shear stress and temperature constraint are applied and seems to be a useful tool to evaluate quality of flour samples (Kahraman et al., 2008).

Principal components analysis (PCA) is a powerful tool for pattern recognition, classification, modelling, and other aspects of data evaluation (Csomos et al, 2002). Also, PCA is a projection method, and dimension reduction of the data can be achieved using a smaller number of principal components than original variables.

The aim of this research is to apply the PCA on a practical example related to treatment of various wheat lots characterized with insect infestation status with inert dust preparations and reveal which treatment showed produced statistically significant impact on the variability of technological Mixolab® parameters of wheat quality.

MATERIAL AND METHODS

In the experiment, wheat variety Danica (*Triticum aestivum* ssp. *vulgare*) were procured from a local producer.

Inert dusts

In the experiment, several types of inert dusts were used: 1) inert dust based on natural zeolite NZ; and 2) two dusts based on diatomaceous earth: DE-S1 and DE-S2, (all originating from Serbia and crudely refined and processed at the Institute for Technology of Nuclear and Other Mineral Raw Materials, Belgrade, Serbia); and 3) a registered product Protect-It® (Hedley Technologies Inc., Canada).

Inert dusts were applied on 0.5 kg wheat samples in five replications, in doses determined in preliminary trials: a) NZ- 1.0 g kg^{-1}; b) DE-S1 and DE-S2- 0.75 g kg^{-1}; and c) standard DE product registered worldwide (Protect-It®) 0.2 g kg^{-1}.

After 21 days of infestation by *S.oryzae* adults, the samples were sieved to separate insects. After total seven weeks of incubation, sieving was repeated to remove the progeny. The samples were than placed into plastic bags and put into refrigerator for 24 h. Next day, sieving was again repeated to remove the rest of possibly remained insects. Sieving was conducted using sieves 7/64'' for insect removal only whereas dust was returned to initial grain mass by mixing for one minute. The sieved samples were stored in plastic bags at room temperature until further examination.

Dough rheological investigation were determined by Mixolab® (Chopin,Tripette et Renaud, Paris, France) which gives dough characteristics during the process of mixing at constant temperature, as well as during the process of heating and cooling. All the measurements were performed using the Mixolab® „Chopin +“ protocol .

The algorithm of PCA can be found in standard chemometric material (Oto, 1999). Descriptive analysis of the data and the PCA were performed using the software package STATISTICA 10.0. In summary, PCA decomposes the original matrix into several products of multiplication into loading (parameters of quality) and score (different treatment with inert dusts) matrices.

Table 1. Experimental design and Mixolab® parameters

	Samples		Wat. abs. (%)	C2 (Nm)	Stab. (min)	α (Nm/ min)	C3 (Nm)	β (Nm/ min)	γ (Nm/ min)	C4 (Nm)	C5 (Nm)
Without insect infestation	1	Control	56,7	0.47	9,83	-0,056	1,79	0,424	-0,07	1,27	1,73
	2	DS S1	57,7	0.48	10,28	-0,082	1,82	0,388	-0,06	1,33	1,86
	3	DS S2	56,6	0.49	10,07	0,028	1,83	0,422	-0,068	1,32	1,83
	4	Prot®	56,5	0.49	10,55	-0,086	1,85	0,378	-0,08	1,38	1,91
	5	NZ	58,2	0.48	10,22	-0,088	1,79	0,398	-0,084	1,27	1,76
With insect infestation	6	I Control	61,2	0.26	4,37	-0,066	1,76	0,528	-0,046	1,44	2,06
	7	I DS S1	60.0	0.50	9,83	0	1,85	0,384	-0,064	1,45	2,03
	8	I DS S2	60,5	0.49	9,88	-0,06	1,83	0,428	-0,046	1,36	1,94
	9	I Prot®	62,4	0.47	7,95	-0,072	1,74	0,436	-0,06	1,25	1,76
	10	I NZ	61,3	0.50	8,33	-0,066	1,83	0,402	-0,058	1,41	1,97

Mixolab® parameters are taken as variables (column of the input matrix) and different treatments with inert dust as mathematical-statistical cases (rows of the matrix) (Table1).

RESULTS AND DISCUSSION

The ranges of average values for Mixolab® parameters for wheat sample were as follows: water absorption value, 56.5-62.4%; weakening of the protein (C2),0.26-0.50 Nm; dough stability, 4.37-10.55 min.; slope α, -0.088-0.028 Nm/min, starch gelatinization (C3), 1.74– 1.85; slope β, 0.378-0.528 Nm/ min; slope γ, -0.084- -0.046 Nm/ min; the stability of the hot-formed gel (C4), 1.25 - 1.45; the starch retrogradation (C5), 1.73 – 2.06 (Table 1).

The number of factors retained in the model for proper classification of the data from Table 2 was determined by application of Kaiser's and Rice's rule (1974). Therefore, two components having eigenvalues >1 were used for further analysis. PCA yields two PCs explaining 75.6% of the total variance in the data. Loading values (i.e. correlation coefficients) higher than 0.7 were marked throughout Table 2 in boldface type.

Table 2. Results of Principal Component Analysis for Mixolab® parameters in grain wheat sample treated with different inert dust

Parameters	**PC1**	**PC2**
Water absorption	**-0,905**	-0,017
C2	**0,863**	0,172
Stability	**0.961**	0,147
α	0,091	0,470
C3	0,516	**0,832**
β	**-0,885**	-0,275
γ	**-0,772**	0,213
C4	-0,466	**0,844**
C5	-0,592	**0,778**
Explained variance	***4,393***	***2,40***
Proportion of total variance %	***48,81***	***26,75***

Projection of the variables on the factorial plane (Fig.1) indicates that the variables dough stability (0.961); water absorption (-0.90), weakening of the protein (C2) (0.86) and slope β (-0.88) most contributed to the first PC indicator (which accounted for 48.81% of the variability), and thus to the total variability of the basic set. The second PC indicator (which accounted for 26.75 % of the variability) is contributed most by starch gelatinization, C3 (0.83) and stability of the hot-formed gel, C4 (0.84).
Parameters correlated with protein component contributed most to the first PC1, and parameters correlated with starch to the second PC2. Water absorption increase in flour from wheat with insect infestation could be attributed to the rise of relative proportion of protein and crude fibre in the damaged kernels as the consequence of insect feeding with starch. Similar was confirmed in the findings of Sanches-Marines et al. (1997). In the study of Korunić et al. (1996), it was found that water absorption of the sample treated with inert dust was raised but the variation was neither correlated to the dose nor was found significant. Although small amounts of inert dusts remain in the flour after wheat treatment (Desmarchelier and Dines, 1987), significant increases in water absorption were found in this study. This could be supported by the fact that inert dusts are known as substances with high moisture

absorption ability (Dakovic et al., 2007) which could contribute to the increase in flour water absorption.
Flour obtained from wheat treated with inert dusts showed higher water absorption and stability during mixing, and less protein weakening during heating in comparison to the control wheat sample non-treated with inert dusts. Changes in the dough rheological properties for flours treated with inert dusts might be partly due to presence of inert dust in the flour. In the study of Korunić et al. (1996), it was found from farinograph and extensigraph results that flour with added inert dust showed increased dough-mixing strength and extensigraph resistance in comparison with the control sample.

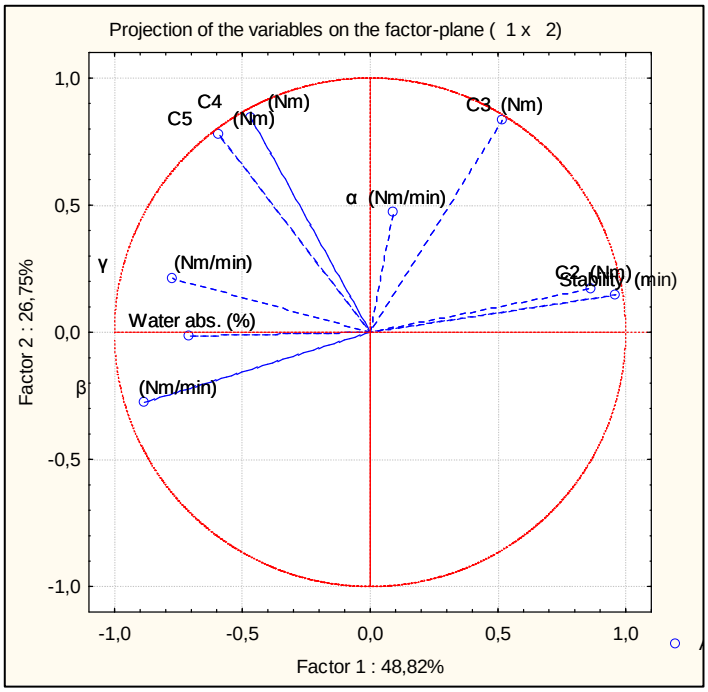

Fig.1. Projection of the variables on the factor-plane

Factor coordinates of individual observations (Fig. 2) indicate that the total variability of the first component is influenced mostly by the non-treated samples with insect infestation (Control) (-5.27). Flour obtained from insect infested wheat treated with inert dusts showed better stability during mixing, and less protein weakening during heating in comparison to the corresponding control wheat (infested, non-treated with inert dusts).The component scores indicated the similarities among the treatments with inert dust. The outlier point is sample Control with insect infestation and without inert dust protection (6).

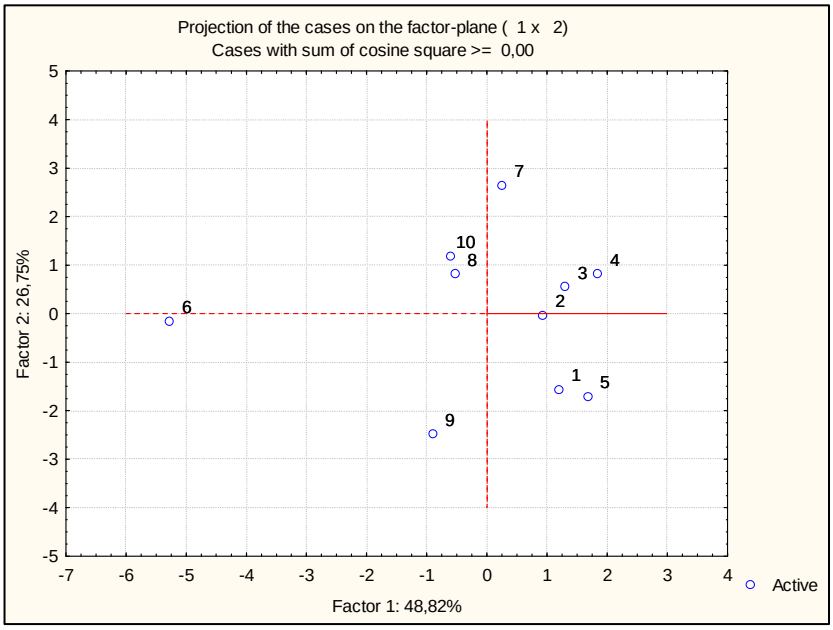

Fig.2. Projection of the cases on the factor-plane

This confirms that the presence of inert dusts may affect the rheological properties of wheat dough although significant changes were not detected by large-deformation tests in the non-infested samples.

CONCLUSION

It could be concluded that PCA is able to point out the outliers among the wheat samples with or without insect infestation and treated with different inert dust. This research showed that parameters correlated with protein component contributed mostly to the first PC1, and parameters correlated with starch to the second PC2. Flour obtained from wheat treated with inert dusts showed higher water absorption and stability during mixing, and less protein weakening during heating in comparison to the control wheat non-treated with inert dusts. The extreme outlier point is control sample with insect infestation and without inert dust protection. Use of inert dusts as protectants in stored wheat grains is reasonable, especially for grains produced in organic farming systems, primarily because the treatments do not compromise food safety.

ACKNOWLEDGEMENT

This work is a part of project funded by research grants III 46005 and III 46008 from the Ministry of Education and Science of the Republic of Serbia.

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QUALITATIVE RESPONSE OF LETTUCE GROWN IN AERATED WATER OF PALIC LAKE

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ABSTRACT: Increasing need among human population for fresh vegetables has generated numerous investigations related to growing alternatives. This research aimed at evaluation of possibility to grow plants on treated wastewater. The growth of *Lactuca sativa* (lettuce) was tested in laboratory conditions, using treated communal and industrial wastewater inflowing from Subotica town into Palic Lake. This water is loaded with organic matter, and as such suitable for plant growth, containing plant nutrients phosphorus, nitrogen, and carbon. The objective was to assess the safety of examined lettuce for human consumption applying controlled conditions, among which most importantly-water aeration. The water used in the experiment contained human pathogens, which were found on the plant samples at the end of the examination period. Total coliform count was analyzed using 3M Petri film test at the beginning of the experiment and at the end of growing period of 30 days.
Results of the experiment indicated that lettuce could be used in ecoremediation technologies for water quality improvement (biomass has increased for 120%), but considering the presence of human pathogens in it, this lettuce would be a risk for human health.
Key words*: lettuce, Palic lake water, nitrogen, phosphorus, pathogenic microorganisms*

INTRODUCTION

Raw food is a main constituent of human diet, nevertheless in modern society nutritionists recommend it as the only constituent. Lettuce (*Lactuca sativa*) is the most widely consumed raw vegetable. It is an annual plant which is being grown worldwide, in different climates and conditions. Lettuce favors high soil humidity and moderately cold weather with temperatures below 24°C for proper growing. Final quality of lettuce is dependant on soil content, quality of irrigation water, ecological factors, and its convenience for human consumption. Natural, non pathogenic, epiphytic microflora is usually present on the lettuce surface after harvesting. But waterborne or soilborn pathogens in growing lettuce can lead to its contamination and consequently be an obstacle in producing safe food.
Nitrogen and phosphorus are necessary nutrients for growth of plants. But, crop production and excessive application of mineral fertilizers has given rise to leaching of these nutrients into the surface waters. High concentrations of nitrogen and phosphorus in surface waters promote the eutrophication and finally lead to perturbation of the ecosystem. There are numerous physical and chemical methods for nutrient removal, but when it comes to the big water systems they are mostly not feasible economically, and have other unfavorable side effects.
Ecoremediation is a biotechnological process that is based on the interaction of plants and microorganisms as an efficient procedure in reducing the consequences of accelerated eutrophication, i.e. degradation of environmental contaminants. Numerous authors emphasize the application of ecoremediation as very convenient

in dealing with organic and inorganic pollution (T. Toyama et. al. 2006, Xian-Ning Li et. al. 2010, L. Stout and K. Nu¨sslein 2010., M. Scholz and Å. Hedmark 2010).
Ecoremediation is economically favorable procedure being 60-80% cheaper than conventional physical and chemical procedures. It has been used to treat wide range of contaminants, especially in shallow waters and waters with low concentration of contaminants. Plant selection is very important, because it directly influences the efficiency of treatment with the high plant resistance, high yields and fast growth (R. P. Singh et. al., 2007.). Choosing the right plant, i.e. varieties with high accumulation potential, is also beneficial regarding the amount of nutrients that can be removed from the wastewater (since nitrogen and phosphorus are essential elements for plant growth) and get transferred into the plant biomass. Besides right plant selection, ecoremediation technologies also use biotechnological approach, i.e. introduction of different bacterial strains. Different researches have shown that certain bacterial strains in plant rhyzosphere promote plant growth and quality. Those are so called Plant Growth Promoting Bacteria (PGPB) and they involve geniuses like: *Acinetobacter, Alcaligenes, Arthrobacter, Azospirillium, Azotobacter, Bacillus, Beijerinckia, Burkholdria, Enterobacter, Erwinia, Flavobacterium, Rhizobium* and *Serrotia* (Bashan and de-Bashan, 2005). PGPB improve plant growth through nutrient supply and maintenance of environmental health. Inoculation of plant roots by PGPB also enhances water remediation.
This research aimed at evaluation of possibility to grow lettuce on treated wastewater, as well as its post usage in human diet. The effect of PGPB on lettuce growth was followed during the examination period of 30 days.

MATERIAL AND METHODS

This research was conducted in laboratory conditions with water sampled from Palic Lake, sector II. The water was analyzed previously to the setting of the experiment, to determine the total inorganic nitrogen, available phosphorus, and the presence of pathogens. Standardized detection methods of spectrophotometer (APHA-Method 4500-NO2-B, Drinking water P-V-2/B) and ionic chromatography (EN ISO 10304 – 1:1995) were used to determine total inorganic nitrogen content. 3M Petri film was used on 37℃ during 48 hours to determine total col iform count. The same method was used at the end of the experiment.
Lettuce seedlings were taken from the local greenhouse that has commercial growing of lettuce. The seedling samples were taken also prior to their planting and tested for total coliforms on 3M Petri film. Plant samples were made separately for lettuce root and above ground part of lettuce by using 20g of each and transferring it into 180ml flasks with peptone solution and homogenized in rotation mixer set at 250rpm for 20 minutes. These samples were tested both at the beginning of the experiment and at the end of examination period.
One of the tested pools with Palic lake water was different comparing to the others and set with lettuce seedlings inoculated with PGPB prior to setting into the water.
Pure cultures of *Azotobacter chroococcum*, *Bacillus circulans*, *Bacillus megaterium, Bacillus pumilus, Bacillus amyloliquefaciens,* and *Bacillus licheniformis* were made in concentration of 10^7 cfu/ml of inoculation medium and used.
Dry matter content was determined by drying the plants on the 80℃ during 3 hours.

RESULTS AND DISCUSSION

The study was intended to provide the assessment of lettuce seedlings' growth. During the experimental period the fresh biomass production was in a range of 45.7% - 182.8%, i.e. 121% on an average (Figure 1.). The experimental pool with PGPB didn't show higher biomass production rate comparing to the experimental

pools without PGPB and was ranging from 36.2%-166.6% of fresh biomass and average value of 122%.

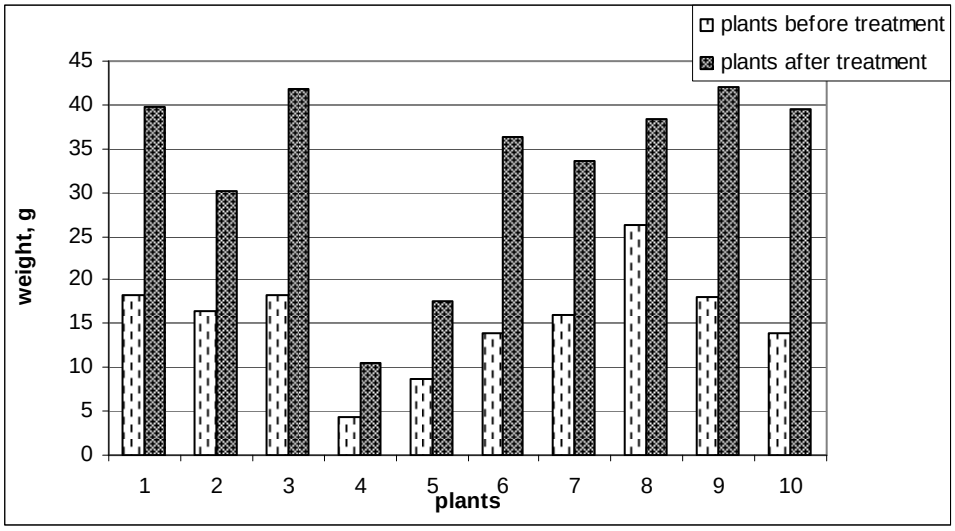

Figure 1. Plant biomass production during experimental period

The results of this research showed that wastewater treatment by lettuce has reduced the nutrient content in the tested water. Total inorganic nitrogen and soluble phosphorus, the essential nutrients for plant growth, were tested at the beginning of the experiment and were ranging from 3 mg/L to 0.11 mg/L, respectively. At the end of the experiment the decrease of nitrogen content was 73.1% and phosphorus content was 34.2% (Figure 2.).

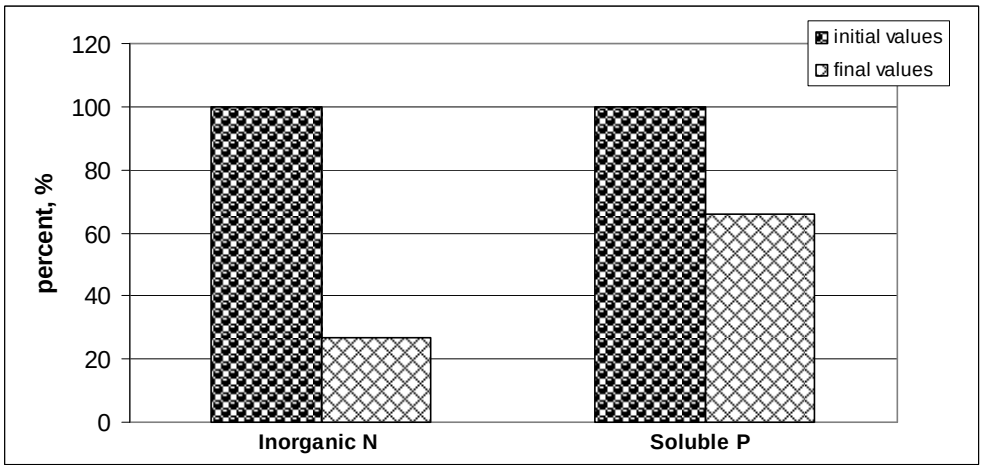

Figure 2. Total inorganic nitrogen content and soluble phosphorus in tested water

In the experimental pool with PGPB enrichment, the content of inorganic nitrogen was reduced by 71.6%, and soluble phosphorus was reduced by 70.7% (Figure 3.). The efficient removal of nitrogen and phosphorus from water was improved by usage of bacterial species that promote immobilization of nitrogen and phosphorus.

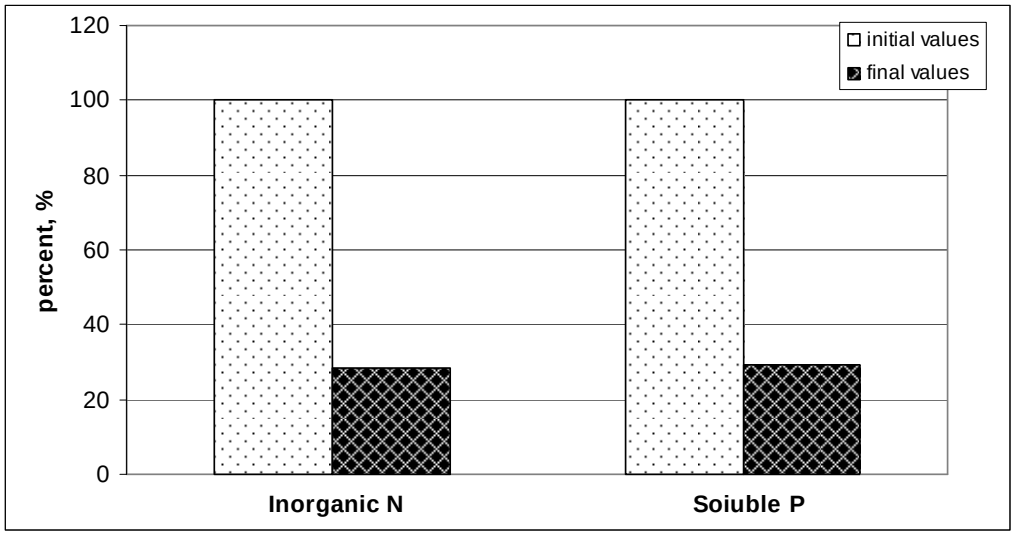

Figure 3. - The content of innorganic nitrogen and soluble phosphorus in the experimental pool with PGPB

Total coliform count in the water was ranging from 225 at the beginning of to 65cfu/ml at the end of experimental period. The lettuce seedlings from greenhouse had a significant count of coliform bacteria in both root and above ground plant. During the experimental period this number was significantly reduced (Table 1.).

Table 1. Total coliform bacteria count in water and plants (CFU/ml)

Tested period (days)	**Total coliforms, at 37℃**		
	water	**root**	**above plant**
0	225	TNTC	4760
15	439	3160	200
30	65	470	100

CONCLUSIONS

The presented study was intended to provide the proof of the possibility to grow lettuce on treated wastewater and its application in phytoremediation, all been proven by the results shown in the tables above and high level of generated biomass (Figure1). This experimental study also proved the benefits of introducing bacterial strains (PGPB) in terms of increased uptake of nitrogen and phosphorus from the water.

Considering the fact that lettuce is used in human diet without previous thermal treatment, i.e. raw, the presence of pathogens in lettuce after the treatment (Table1.) indicates its inconvenience for human consumption.

Further investigation need to be done in order to determine the transport mechanisms in lettuce of pathogen from its roots to above ground parts, as well as its total degradation by specific PGPB strains application and different plant selection.

ACKNOWLEDGEMENTS

This paper is part of the research that has been conducted under the Project 31080 financed by the Ministry of Science and Technological Development, Republic of Serbia.

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STUDY OF LIPASE-CATALYZED SYNTHESIS OF ASCORBYL OLEATE USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT: L-ascorbic acid has good antioxidative properties but its efficiency in stabilizing fats and oils in products with high lipid content is negligible due to its hydrophilic characteristics. On the other hand, fatty acid ascorbyl esters are liposoluble, with even better antioxidative properties comparing to L-ascorbic acid. Therefore, developing of industrial process for lipase-catalyzed synthesis of vitamin C fatty acid esters, considering numerous advantages over conventional chemical methods (mild reaction conditions, high regioselectivity, and simplified downstream processing), is of great interest. In this study, L-ascorbyl oleate was synthesized in esterification reaction between vitamin C and oleic acid catalyzed by immobilized lipase from *Candida antarctica* in acetone as a reaction medium. Response surface methodology and 5-level-5-factor central composite rotatable design were employed in order to investigate interactions between experimental factors (initial water content, temperature, substrates molar ratio, vitamin C concentration, and enzyme amount), determine their individual influence on molar conversion, and eventually optimize the synthesis. Based on the experimental data, regression model, expressed with second order polynomial equation, was obtained. At values in the range of examination, enzyme amount had no influence on conversion so it was fixed at the minimum (0.2 % (w/v)). The maximum molar conversion of 91.3 % was predicted and corresponding, optimal reaction conditions were: temperature – 60 ºC, initial water content – 0 % (v/v), vitamin C concentration – 0.02 M, and substrate molar ratio – 1:15. Our system provided reaction conditions which enabled high conversions, thus obtained results may be used as a starting point for the process scale-up.
Keywords: *ascorbyl oleate, lipase, optimization, response surface methodology*

INTRODUCTION

Fats and oils are prone to autooxidation which causes shortening of the shelf life of food and cosmetics containing them. As additives in such products, synthetic antioxidants (BHT and BHA) are being used to prevent deterioration of lipids, but their potential toxicity is the matter of concern (Karmee, 2009). Therefore, natural, non-toxic antioxidants which have high free radical scavenging capacity and liposolubility, such as fatty acid ascorbyl esters, are preferable for this usage. Currently, ascorbyl palmitate is being industrially produced in process catalyzed by chemical means, despite disadvantages such as low yields and absence of regioselectivity, thus complicated downstream processing (Yan et al., 1999). Also, application of ascorbyl palmitate is limited due to its low solubility in oils which could be overcome by using unsaturated fatty acid esters of vitamin C. Nowadays, subject of many researches is development of industrial process for enzymatic synthesis of unsaturated fatty acid ascorbyl esters in which lipases would be used as a catalyst. Such production would provide avoidance of the main obstacles of chemically catalyzed synthesis and it would be classified as environmentally friendly.

Lipases (tryacylglycerol acylhydrolases, E.C. 3.1.1.3.), besides their application in hydrolysis of triglycerides, are widely used as a biocatalysts in esterification, transesterification, and interesterification reactions (Bezbradica et al., 2009). Fatty acid ascorbyl esters were so far synthesized by using several microbial lipases: *Candida antarctica* lipase, type B (Humeau et al., 1998a; Humeau et al., 1998b; Lerin et al., 2010; Viklund et al., 2003), lipase from *Thermomyces lanuginosus* (Reyes-Duarte et al., 2011), *Bacillus stearothermophylus* SB1 lipase (Bradoo et al., 1999), and lipase from *Rhizomucor miehei* (Burham et al., 2009). Oils, saturated and unsaturated fatty acids, and their methyl and vinyl esters were previously applied, whereas higher yields were achieved when more expensive, activated acyl donors were used (Karmee, 2009). Solvents with high log P values, which are most commonly used as a reaction medium in lipase catalyzed esterifications, have proved to be unsuitable for FA ascorbyl esters biosynthesis because of the high polarity of vitamin C (Song and Wei, 2002). Hence, reaction was mostly conducted in more polar solvents such as *t*-amyl alcohol, *t*-butanol, and acetone. Ionic liquids were also successfully applied, although its usage decreased enzyme stability and increased price of the process (Adamczak and Bornscheuer, 2009). In the majority of studies, it was reported that control of the initial water content, excessive amount of acyl donor, and water removal by molecular sieves were good tools for moving equilibrium towards the product (Karmee, 2009). Optimum temperature varied from 30 to 65 ºC in previous researches, indicating that this parameter is strongly affected by properties of substrates, product, and solvent (Song and Wei, 2002; Chang et al., 2009; Lv et al., 2008). At the present time, efforts are being made in order to optimize lipase-catalyzed synthesis of ascorbyl oleate since it has strong antioxidative effects (stronger then vitamin C and ascorbyl palmitate), its solubility in fats and oils is high, and oleic acid, as an acyl donor, can be classified as inexpensive and commercially available.

The main goal of this research was to optimize esterification of vitamin C and oleic acid catalyzed with immobilized lipase from *C. antarctica* in order to maximize limiting substrate molar conversion. Acetone was used as a reaction medium due to its low price, GRAS status, and high volatility. The effects of key experimental factors (enzyme loading, vitamin C concentration, temperature, initial water content, and substrate molar ratio) on reaction progress, as well as their interactions, were examined by applying response surface methodology (RSM) and 5-level-5-factor central composite rotatable design (CCRD).

MATERIAL AND METHODS

Enzyme and chemicals

Novozym® 435 (lipase from *C. antarctica*, type B immobilized on acrylic resin) was purchased from Novozymes (Bagsvaerd, Denmark). Substrates were L-ascorbic acid (99.7 %, Zorka, Šabac, Serbia) and oleic acid (Ph. Eur., NF pure) purchased from AppliChem, Darmstadt, Germany. As reaction media acetone (99.5 %, Zorka Pharma, Šabac, Serbia) was utilized. Substances used for the quantitative HPLC analyses were methanol obtained from JT Baker, USA and phosphoric acid purchased from Sigma-Aldrich Chemie Gmbh, both of HPLC grade.

Procedure for the enzymatic synthesis

Experiments were carried out in 100 ml capped vessels. The reaction mixture was composed of different amounts of vitamin C, oleic acid, enzyme, water, and acetone and the total volume was 10 ml. The reactions were carried out in a shaker at 250 rpm and at temperatures in range from 40 to 60 ℃. In control samples (lipase not added), which were prepared by exposure to the same temperature treatment, product was not detected. All experiments were conducted in duplicate and all

standard deviations were less than 5 %. In further calculations, average values were used.

Experimental design and statistical analysis

A 5-level-5-factor CCRD, including 32 experimental points of which 16 factorial, 10 axial, and 6 center, was employed in this study. Coded and actual levels of variables, experimental design, and obtained results are presented in Table 1.

Table 1. Experimental design (coded levels and corresponding actual values of variables) and obtained conversions

Treatment	Water content, X_1(%(v/v))	Temperature, X_2(°C)	Enzyme amount, X_3(%(w/v))	Substrates molar ratio, X_4	Vitamin C concentration, X_5(mol dm^{-3})	Conversion, Y(%)
1	1 (0.15)	1 (55)	1 (0.8)	1 (1:12)	1 (0.17)	13.89
2	1 (0.15)	1 (55)	1 (0.8)	-1 (1:6)	-1 (0.07)	35.90
3	1 (0.15)	1 (55)	-1 (0.4)	1 (1:12)	-1 (0.07)	44.05
4	1 (0.15)	1 (55)	-1 (0.4)	-1 (1:6)	1 (0.17)	18.22
5	1 (0.15)	-1 (45)	1 (0.8)	1 (1:12)	-1 (0.07)	32.35
6	1 (0.15)	-1 (45)	1 (0.8)	-1 (1:6)	1 (0.17)	11.53
7	1 (0.15)	-1 (45)	-1 (0.4)	1 (1:12)	1 (0.17)	7.41
8	1 (0.15)	-1 (45)	-1 (0.4)	-1 (1:6)	-1 (0.07)	29.79
9	-1 (0.05)	1 (55)	1 (0.8)	1 (1:12)	-1 (0.07)	56.97
10	-1 (0.05)	1 (55)	1 (0.8)	-1 (1:6)	1 (0.17)	22.78
11	-1 (0.05)	1 (55)	-1 (0.4)	1 (1:12)	1 (0.17)	16.44
12	-1 (0.05)	1 (55)	-1 (0.4)	-1 (1:6)	-1 (0.07)	48.96
13	-1 (0.05)	-1 (45)	1 (0.8)	1 (1:12)	1 (0.17)	9.35
14	-1 (0.05)	-1 (45)	1 (0.8)	-1 (1:6)	-1 (0.07)	30.12
15	-1 (0.05)	-1 (45)	-1 (0.4)	1 (1:12)	-1 (0.07)	44.77
16	-1 (0.05)	-1 (45)	-1 (0.4)	-1 (1:6)	1 (0.17)	20.12
17	2 (0.2)	0 (50)	0 (0.6)	0 (1:9)	0 (0.12)	21.77
18	-2 (0)	0 (50)	0 (0.6)	0 (1:9)	0 (0.12)	31.65
19	0 (0.1)	2 (60)	0 (0.6)	0 (1:9)	0 (0.12)	32.16
20	0 (0.1)	-2 (40)	0 (0.6)	0 (1:9)	0 (0.12)	27.99
21	0 (0.1)	0 (50)	2 (1)	0 (1:9)	0 (0.12)	25.09
22	0 (0.1)	0 (50)	-2 (0.2)	0 (1:9)	0 (0.12)	25.28
23	0 (0.1)	0 (50)	0 (0.6)	2 (1:15)	0 (0.12)	18.64
24	0 (0.1)	0 (50)	0 (0.6)	-2 (1:3)	0 (0.12)	17.18
25	0 (0.1)	0 (50)	0 (0.6)	0 (1:9)	2 (0.22)	9.24
26	0 (0.1)	0 (50)	0 (0.6)	0 (1:9)	-2 (0.02)	35.29
27*	0 (0.1)	0 (50)	0 (0.6)	0 (1:9)	0 (0.12)	23.93
28*	0 (0.1)	0 (50)	0 (0.6)	0 (1:9)	0 (0.12)	28.17
29*	0 (0.1)	0 (50)	0 (0.6)	0 (1:9)	0 (0.12)	27.56
30*	0 (0.1)	0 (50)	0 (0.6)	0 (1:9)	0 (0.12)	25.84
31*	0 (0.1)	0 (50)	0 (0.6)	0 (1:9)	0 (0.12)	22.93
32*	0 (0.1)	0 (50)	0 (0.6)	0 (1:9)	0 (0.12)	29.17

*Central point

All 32 experiments were conducted randomly so that the possibility of systematic errors would be excluded. Response surface regression (RSREG) method was employed for analyzing of experimental data in order to fit them to the second-order polynomial equation

$$Y = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki} X_i + \sum_{i=1}^{5} \beta_{kii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{kij} X_i X_j \quad (1)$$

where Y is response (conversion), β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant regression coefficients, X_i and X_j are uncoded independent variables, and k is the number of single factor. MATLAB 7.0 (Mathworks Inc., Natick, MA, USA) was used when the least square method was applied for the response function coefficients calculation

and their statistical significance estimation. Into the final model, only significant terms with $p \leq 0.05$ were included. Fisher test was used in order to validate adequacy of the obtained model and student distribution was employed for the evaluation of the coefficients significance.

HPLC analysis

Quantitative analysis of reactants and products were conducted on Akta Purifier HPLC system. Waters Spherisorb ODS 2-C18, 250 mm × 4.6 mm, 5 µm reverse phase column was employed. Reaction mixture (10 µl), fifteen times diluted was injected and eluted with 1 ml min^{-1} of Methanol/H_3PO_4, 100/0.1 (v/v). Ascorbyl oleate was detected by UV detector at 235 nm.

RESULTS AND DISCUSSION

Adequacy of the model was confirmed by Fisher test (F_r value was 4.1 which is lower then theoretical (4.58) for significance level of 5 %). Student test was employed for determining the significance of the regression coeficients and based on it, β_0, β_1, β_2, β_4, β_5, β_{11}, β_{22}, β_{44}, β_{15}, β_{25}, and β_{45} were included in model. Following regression model was obtained:

$$Y = 26.17 - 3.17X_1 + 3.34X_2 + 0.45X_4 - 10.6X_5 + 0.737X_1^2 + 1.58X_2^2 - 1.47X_5^2 + 1.33X_1X_5 - 1.63X_2X_5 - 3.7X_4X_5 \quad (2)$$

It can be noticed that among linear terms, only the one describing effect of enzyme amount (β_3) was not significant, indicating that lipase loading, in the range from 0.2 to 1 % (w/v), was not affecting conversion degree. Positive quadratic regression coefficients β_{11} and β_{22} (functions with minimum) and negative, β_{44}, (function with maximum) were significant, as well as the terms that were describing negative interaction of vitamin C concentration with temperature (β_{25}) and substrates molar ratio (β_{45}) and positive interaction between ascorbic acid concentration and initial water content (β_{15}). From the Table 1, it can be observed that the highest conversion of 56.97 % was achieved in experiment No. 9. Response surface plots showing influence of pairs of factors on molar conversion are represented in figures.

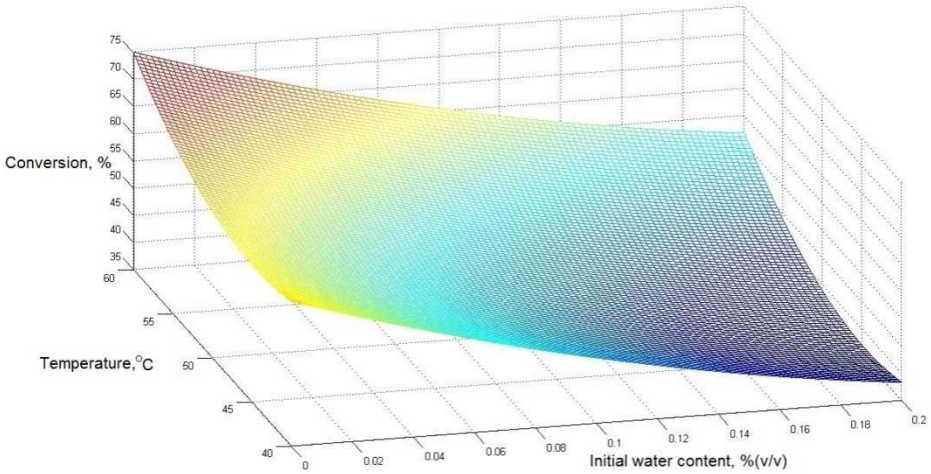

Figure 1. Response surface plot showing interactions between temperature and initial water content

Within the entire observed range, molar conversion increased with decrease of initial water content (minimum of this function was out of scope) reaching maximum when water was not added (Figs. 1 and 2). Obtained result indicated that additional water was not needed for keeping immobilized lipase in its active form. Influence of reaction temperature was also described by the function with minimum out of the monitored range (Fig. 1). Increase of temperature up to the 60 ºC (higher temperatures were not tested since they would cause boiling of reaction mixture) had positive effect on the conversion degree. Impact of vitamin C concentration was represented by linear function with negative slope. Hence, maximum conversions were achieved at lowest concentrations of L-ascorbic acid. From figure 2, in which mutual effect of initial water content and substrates molar ratio (parameters X_2, X_3, and X_5 were fixed at 0 levels) is represented, it can be noticed that maximum molar conversion was reached at 1:10 ratio. However, position of local maximums depends significantly on vitamin C concentration owing to strong negative interaction between factors X_4 (substrates molar ratio) and X_5 (ascorbic acid concentration), which is depicted in Fig. 3. At high initial ascorbic acid concentration, minimum substrates molar ratio (1:2) was optimal and *vice versa* (maximum molar ratio of 1:15 was optimal at low concentrations of vitamin C). Conversion decrease at highest oleic acid contents was probably induced by mass-transfer limitations caused by its high viscosity (Burham et al., 2009). Since best results were achieved at 0.02 mol dm^{-3} of limiting substrate, molar ratio 1:15 was set as optimal. Lipase loading was fixed at the minimum (0.2 % (w/v)) considering that it was not significant. Maximum molar conversion of 91.3 % was predicted by the model at optimized reaction conditions, which is significantly higher than highest conversion, accomplished in experiment No.9.

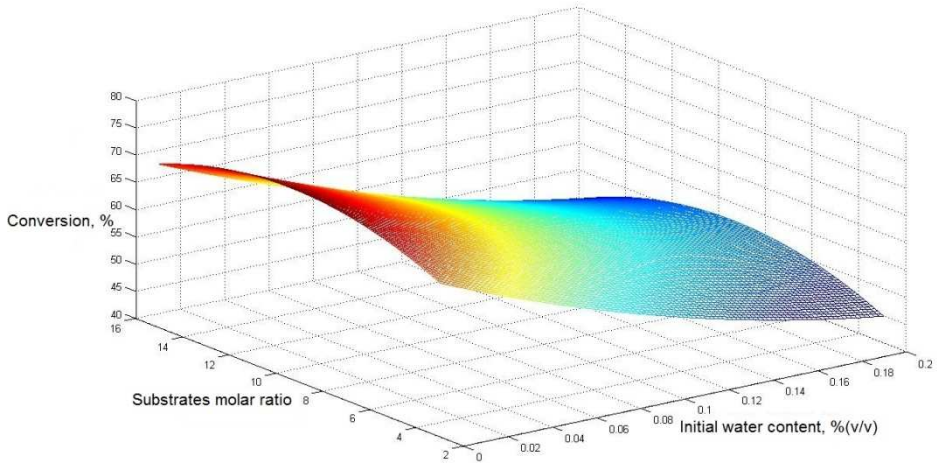

Figure 2. Response surface plot showing interactions between substrates molar ratio and initial water content

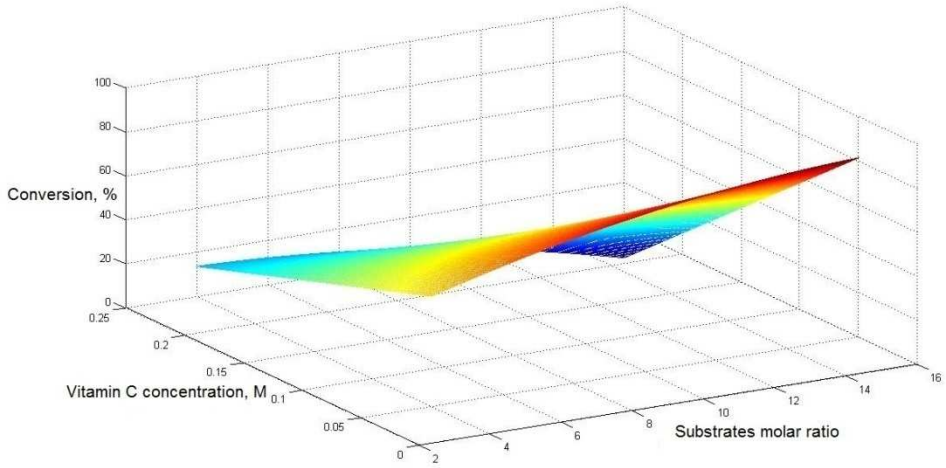

Figure 3. Response surface plot showing interactions between vitamin C concentration and substrates molar ratio

CONCLUSIONS

Aim of this study was to optimize enzymatic synthesis of ascorbyl oleate in acetone with vitamin C and oleic acid as substrates by applying response surface methodology (RSM) and 5-level-5-factor central composite rotatable design (CCRD). Results of the research are comparable with previously reported with more expensive solvents or activated acyl donors. Hence, it is possible to use them in a further process optimization on larger scales.

ACKNOWLEDGEMENTS

The authors are grateful for financial support from Serbian Ministry of Science (project III 46010).

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THE INFLUENCE OF REACTION CONDITIONS ON THE HYDROLYSIS OF NATIVE CORN, POTATO AND PEA STARCHES

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ABSTRACT: Enzymatic modification of starch provides a good solution for improving the processing behaviour and stability of starches. The composition of starch, especially in terms of amylose and amylopectin, is the most researched aspect of influence on functional behaviour of starch. Granular structure and extension of crystalline and amorphous zones have direct impact on starch properties. Using of amylolytic enzymes below the gelatinization temperature leads to a partial degradation of the suspended starch granules with retention of the grain structure.
In this study the influence of reaction conditions (type of starch, type of enzymes, reaction temperature and reaction time) on the grade of enzymatic hydrolysis of native corn, potato and pea starches, which differ according to the starch-type (A, B and C-type starches) was researched. The progress of the enzymatic reaction was followed by determination of glucose content in the supernatant (converted to starch) at specified reaction temperatures (30, 40, 50 and (55)60 ℃) and in specified time intervals (20, 44, 68, 92 h). It was found that the degree of hydrolysis of potato starch showed significantly lower values than that of corn and pea starch. The highest glucose values were determined after degradation with amyloglucosidase (Dextrozyme). By the hydrolysis of potato starch, glucose content in supernatant was below 10 %. Corn and pea starches were attacked most easily (to 60 %) in comparison to potato starch. Higher temperature and longer hydrolysis time led to a greater degradation of native starches.
Key words: *pea starch, corn starch, potato starch, enzymatic hydrolysis, enzymatic modification, hydrolysis conditions*

INTRODUCTION

Modified starch plays a significant role in rheological/texture properties of food and has significant industrial applications. In food processing modified starches are used as a thickener, stabilizer, gelling agent, bulking agent and water retention agent. Those properties can be improved by physical, chemical or enzymatic modification of starch granule. Traditional acid hydrolysis of starch to glucose is being superseded by enzymatic processes because acid processes leads to undesirable by-product. The fact that some enzymes have a particular capacity in transforming starch to shorter polymers inspired several researches in the recent years (Planchot, et al., 1994; Shariffa et al., 2009, Zieba et al., 2011; Gularte and Rosell, 2011). The importance of the enzymatic liquefaction of raw starch without heating has been well recognised, mainly due to energy savings which reduces the overall cost of starch processing (Robertson et al., 2006). This has generated a worldwide interest in the discovery amylases that directly hydrolyse raw starch in a single step at moderate temperature, much below the gelatinization temperature (Uthumporn et al., 2010). Many studies have shown that rate of hydrolysis of starch granules strongly depends on the botanical source from which they originated and that it mainly occurs in amorphous region of starch granule (Lacerda et al., 2008, Shariffa et al., 2009; Ahmed and Auras, 2011; You, S. and Izydorczyk, M.S., 2007).
The objective of this study was to investigate the influence of reaction conditions by the enzymatic hydrolysis of native corn, pea and potato starches in its native (raw)

granular state and compare the influence of two amylolytic enzymes and their ability to hydrolyse these starches.

MATERIALS AND METHODS

Materials: Raw corn and potato starch (Maisita 2100740 and Stärkina 20000-50) were obtained from the Agrana Group. Pea starch was isolated from peas at the Institute of Food Technology, University of Live Sciences, Vienna.
Enzymes: The commercial enzymes used in the present study, *Dextrozyme 225/75L* (*Aspergillus niger* glucoamylase and *Bacillus acidopullulyticus* pullulanase) and *Promozyme 200L* (*Bacillus acidopullulyticus* pullulanase) were products of Novo Industry (Novo, Denmark).
Determination of moisture content: The moisture content of starch samples were determined by using IR-30 Moisture Analyser (Sartorius thermo control DTC 01L, Haack, Vienna). Starch (5g) were spread uniformly on the pan and heated at 105℃.
Starch hydrolysis: Raw starch solution (20%) was prepared in distilled water (pH was adjusted to 4.5 with citric buffer) and incubated with Dextrozyme 225/75L or Promozyme 200L (10 times higher enzyme concentration as recommended by producer) at different temperatures (30, 40, 50 and 60(55) ℃). In the experiments with the potato starch were used temperatures no higher than 55 ℃, because the temperature of 60 ℃ leads to its gelatinization. A fter 20, 44, 68 and 92 hours 3 ml of suspension were removed from the water bath with a transfer pipette and centrifuged to obtain clear supernatant. Reducing sugars were analysed by 3,5-dinitrosalicylic acid (DNS) method (Sumner and Somers, 1949) using glucose as a standard. Each analysis was performed in duplicate. Suitable controls such as starch control (without enzyme) and enzyme control (without substrate) were determined. Degree of hydrolysis (%) was calculated as follows:

$$Glucose\ (\%) = \frac{\text{g reducing sugar expressed as glucose}}{\text{g total starch dry weight}}\ \text{x}\ 100$$

RESULTS AND DISCUSSION

Use of amylolytic enzymes below the gelatinization temperature of the starch results partial hydrolysis of the suspended starch granules with retention of the grain structure. The degree of starch hydrolysis was determined by measuring glucose content in supernatant at different time intervals. Figures 1 to 6 and Table 1 show the course of glucose production during enzymatic degradation of native corn, potato and pea starch with Dextrozyme and Promozyme at several reaction temperatures.

By corn starch (Figures 1 and 2, Table 1), after 92 h hydrolysis, glucose levels were 42.67% (measured at 40 ℃), 52.62% (at 50 ℃) and 5 2.52% (at 55 ℃). At 60 ℃ the glucose values were lower than at 40 or 55 ℃ (49.6 5%). The hydrolysis of corn starch with Promozym showed much lower values for the glucose content, which was after 92 h degradation between 0.09 to 0.22% .

Neither 40 ℃ nor 50 ℃ and 55 ℃ were sufficient t o raise the glucose level by potato starch above 10% in the hydrolysis with Dextrozyme (Figures 3 and 4, Table 1). The hydrolysis of potato starch with Promozym led to very low glucose levels at all reaction temperature, the glucose values were only 0.11 to 0.25%.

The pea starch hydrolysis showed a similar trend as the corn starch (Figures 5 and 6, Table 1). The higher values at 40℃ indicate a more intense hydrolyse of pea starch with Dextrozyme. After a 92-hour hydrolysis they amounted to 60.36%. A further

increase of reaction temperature to 50 or 60 ℃ gave lower values for the glucose content, and after 92 h hydrolysis it was 44.83% and 31.55% respectively. The effect of temperature on the degradation of pea starch with Promozym was similar to the degradation of Dextrozyme (the highest values were measured at 40 ℃), but with a much lower glucose content. The values after 92 h, were 1.35% (at 30 ℃), 2.66% (at 40 ℃) and only 0.54% glucose (at 60 ℃).

Table 1. Glucose content (% based on starch) after hydrolysis of corn (M), potato (K) and pea (E) starches

Sample	Starch type	Enzymes	Temperature (℃)	Glucose content[1) 2)] (%)			
				20	44	68	92
M-Dex-30 ℃	CORN STARCH	Dextrozyme (amyloglucosidase+pullulanaze)	30	17,65 ± 0,15	26,97 ± 0,05	31,78 ± 0,20	35,78 ± 0,05
M-Dex-40 ℃			40	29,32 ± 0,03	33,15 ± 0,07	35,78 ± 1,01	42,67 ± 0,30
M-Dex-50 ℃			50	31,95 ± 0,06	40,31 ± 1,43	50,59 ± 1,60	52,62 ± 0,32
M-Dex-55 ℃			55	34,65 ± 0,30	40,97 ± 1,60	48,00 ± 0,81	52,52 ± 0,30
M-Dex-60 ℃			60	37,31 ± 0,41	42,24 ± 0,41	45,16 ± 1,11	49,65 ± 0,60
M-Pro-30 ℃		Promozyme (pullulanase)	30	0,12 ± 0,00	0,16 ± 0,00	0,14 ± 0,00	0,14 ± 0,00
M-Pro-40 ℃			40	0,13 ± 0,09	0,15 ± 0,02	0,18 ± 0,01	0,19 ± 0,01
M-Pro-50 ℃			50	0,12 ± 0,02	0,19 ± 0,01	0,19 ± 0,07	0,23 ± 0,07
M-Pro-55 ℃			55	0,04 ± 0,00	0,08 ± 0,00	0,11 ± 0,01	0,14± 0,09
M-Pro-60 ℃			60	0,04 ± 0,01	0,05 ± 0,00	0,06 ± 0,00	0,09 ± 0,01
K-Dex-30 ℃	POTATO STARCH	Dextrozyme (amyloglucosidase+pullulanaze)	30	1,45 ± 0,01	1,99 ± 0,02	2,50 ± 0,05	2,90 ± 0,01
K-Dex-40 ℃			40	4,35 ± 0,23	6,80 ± 0,11	8,10 ± 0,17	9,37 ± 0,35
K-Dex-50 ℃			50	2,59 ± 0,06	3,88 ± 0,06	4,67 ± 0,07	5,18 ± 0,08
K-Dex-55 ℃			55	3,18 ± 0,02	3,50 ± 0,08	3,87 ± 0,07	4,00 ± 0,03
K-Pro-30 ℃		Promozyme (pullulanase)	30	0,06 ± 0,00	0,07 ± 0,01	0,09 ± 0,00	0,11 ± 0,03
K-Pro-40 ℃			40	0,14 ± 0,012	0,19 ± 0,00	0,22 ± 0,00	0,25 ± 0,00
K-Pro-55 ℃			55	0,11 ± 0,001	0,18 ± 0,01	0,21 ± 0,01	0,24 ± 0,08
E-Dex-30 ℃	PEA STAARCH	Dextrozyme (amyloglucosidase+pullulanaze)	30	8,33 ± 0,42	13,93 ± 0,59	22,22 ± 0,06	28,59 ± 0,06
E-Dex-40 ℃			40	31,51 ± 0,07	44,69 ± 0,32	54,86 ± 0,08	60,36 ± 0, 07
E-Dex-50 ℃			50	21,66 ± 0,32	32,53 ± 0,82	40,37 ± 1,26	44,83 ± 1,01
E-Dex-60 ℃			60	14,39 ± 0,25	27,77 ± 0,03	29,19 ± 0,06	31,55 ± 0,90
E-Pro-30 ℃		Promozyme (pullulanase)	30	0,60 ± 0,09	0,78 ± 0,03	1,02 ± 0,01	1,35 ± 0,07
E-Pro-40 ℃			40	1,20 ± 0,03	1,46 ± 0,00	2,18 ± 0,02	2,66 ± 0,00
E-Pro-60 ℃			60	0,14 ± 0,01	0,26 ± 0,01	0,45 ± 0,00	0,54 ± 0,01

[1)] *Values are expressed as mean ± SD of four repeats*
[2)] *to the blank corrected glucose content*

Influence of reaction temperature: Lower degree of starch degradation at higher reaction temperature (50, 55 and 60 ℃) is related to adsorption of the enzyme on substrate (Karakatsanis et al., 1997). As indicated, enzymes adsorb better on substrate at lower temperatures, while higher temperatures accelerate desorption and assumption is that better adsorbed enzyme may develop a better effect. Another possible explanation for poorer starch hydrolysis at higher temperature (above 40℃) would be the binding of amylose in an amylose-lipid complex (Anger et al. 1994), wherefore the access of enzyme to amylase molecules is denied and it affects the final degree of hydrolysis.

Influence of the enzyme type: Numerous studies showed that the addition of pullulanase to glucoamylase or to α-amylase accelerated the degradation of native starch (Ueda and Marshall, 1980; Sprinat and Antranikian, 1992).
In this study Promozym 200L (pullulanase) and Dextrozyme 222/75L (pullulanase in combination with gluco-amylase) were used. These are two basic types of enzymes: (1) an endo enzyme (pullulanase), which cleaves the α-1, 6-glucosidic linkages of amylopectin, creating two smaller molecules, and (2) an exo-enzyme (gluco-amylase) which cleaved the α-1,4 - and α-1,6-glucosidic bonds, which resulted in the

glucose monomers. As expected, with Dextrozyme was achieved better starch degradation rate than with Promozym (pullulanase alone). It can be explained with a poor adsorption of pullulanase to starch granules (Wang et al., 1996).

Influence of starch type: In this study we used corn, potato and pea starch, which differ according to the starch-type (A, B, and C-type) (Imberty et al., 1988; Imberty and Pérez, 1988). It was found that the degree of hydrolysis of potato starch was significantly lower than that of maize or pea starch. Compared with pea starch, corn starch was attacked most easily. Only at a reaction temperature of 40 ℃ higher glucose values was obtained for pea starch than for the corn starch. Corn, pea and potato starch can be very slightly degraded with Promozym alone. Only in the pea starch slightly higher glucose content was observed.
From the literature is known that A-type starches are hydrolysed better than B-type (Gallant et al, 1982). The cereal starches, which have A-type crystallinity, have been degraded better than the legume starch having C-structure. The potato starch, which is a B-type, was hydrolysed the lowest. The differences between corn and pea starch can be explained that molecules of corn starch are packed in the less dense crystalline regions and therefore are more accessible to enzymatic attack, than in the starches with higher amylose content as is pea starch (Hoover and Sosulski, 1985a, Uthumporn et al., 2010). Poorer hydrolyse of potato starch in comparison to other types of starches could be explained by the size of potato starch granules which are relatively large and have smaller total surface area (Shariffa et al., 2009, Zieba et al., 2011). The results of starch hydrolysis confirmed that the susceptibility was affected by the botanical origin, and amylose/amylopectin ratio and degree of starch crystallinity.

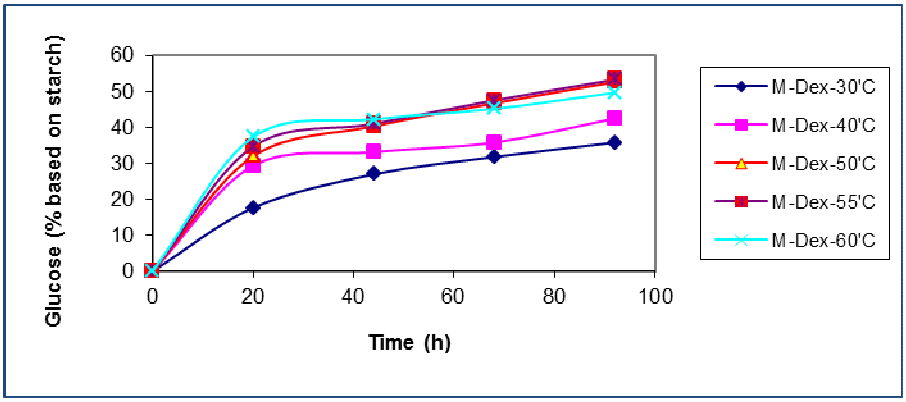

Figure 1. Glucose content (% based on starch) after hydrolysis of corn starch with Dextrozyme

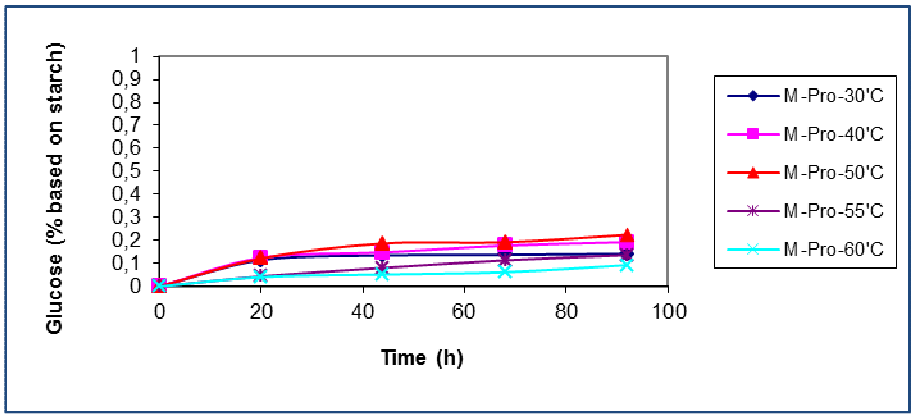

Figure 2. Glucose content (% based on starch) after hydrolysis of corn starch with Promozyme

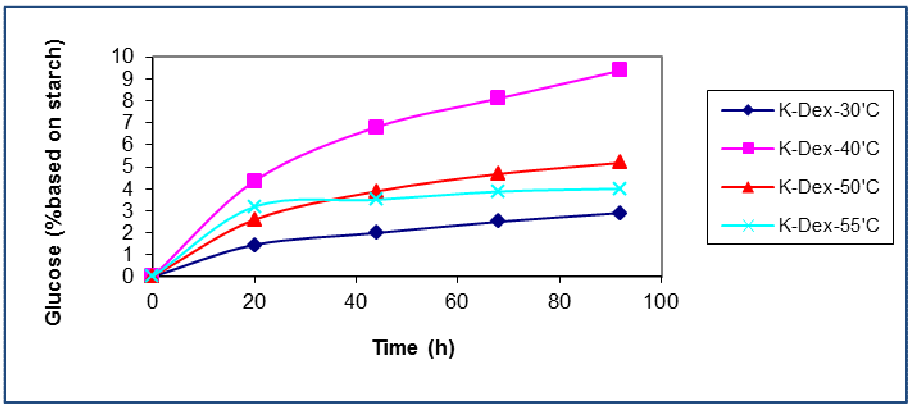

Figure 3. Glucose content (% based on starch) after hydrolysis of potato starch with Dextrozyme

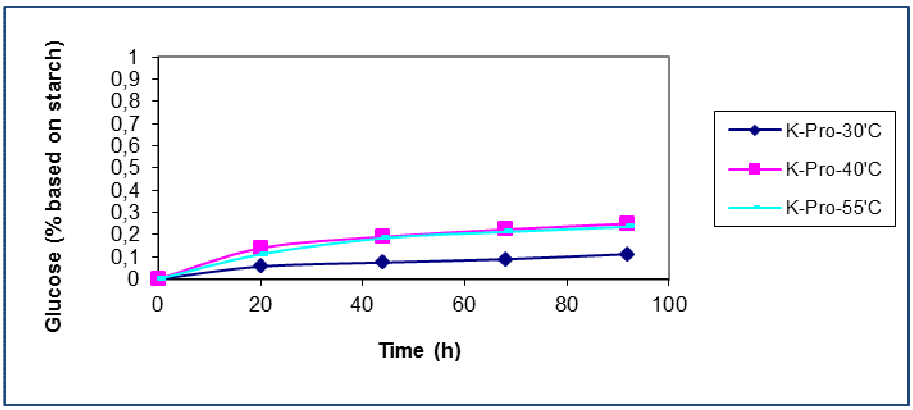

Figure 4. Glucose content (% based on starch) after hydrolysis of potato starch with Promozyme

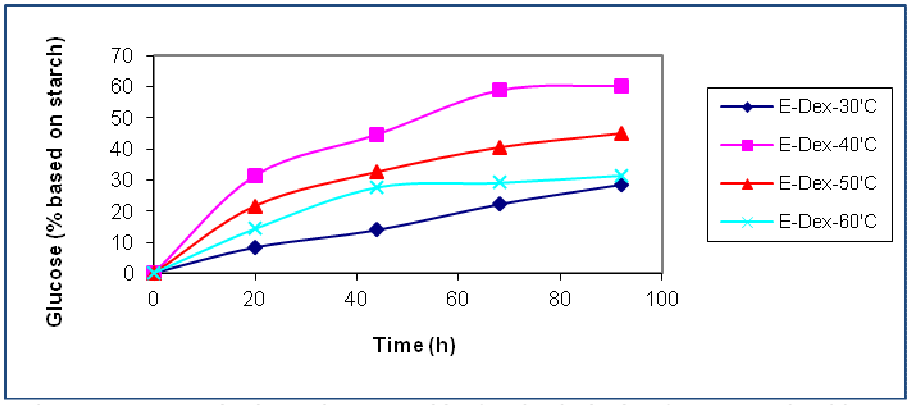

Figure 5. Glucose content (% based on starch) after hydrolysis of pea starch with Dextrozyme

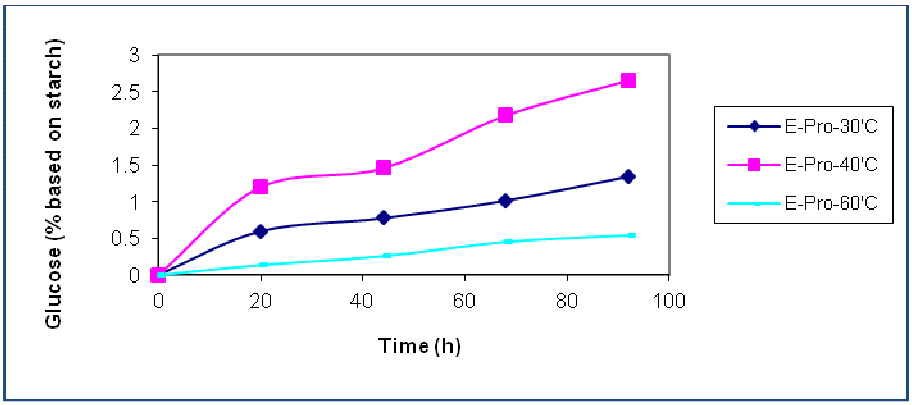

Figure 6. Glucose content (% based on starch) after hydrolysis of pea starch with Promozyme

CONCLUSION

Amylolitic enzymes, combination of gluco-amylase and pullulanase, are able to hydrolyse granular starch at a sub- gelatinization temperature in relatively high degree. Significant differences in a degradation of starches from corn, potato and pea were observed. The highest glucose values were determined after hydrolysis of corn starch and the lowest after hydrolysis of potato starch. Longer hydrolysis time and higher temperature (to 40℃) affects the hydrol ysis rate positively. This research clarified that hydrothermal enzymatic modification of starches was very strong affected by hydrolyse condition and that with their variation can be obtained modified starches which can have a specific application in the food industry.

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IMPACT OF QUANTITY AND QUALITY OF WET GLUTEN ON HARD BISCUITS PROPERTIES

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ABSTRACT: Dimensions of individual biscuit pieces such as height, width and length thereof are a significant factor in the quality of hard biscuits. Provided that these dimensions are uniform, it would possible to apply an automatic packing of desired weights. Dimensions of hard biscuits largely depend on the quality of flour and dough, which is closely related to the content and quality of gluten in the flour. The object of this study was to analyze the impact of quantity and quality of wet gluten on the physical, chemical and on sensory properties of hard biscuits. Three white wheat flour samples containing 20, 22 and 24% of wet gluten were used in this study. The quality of flour samples in terms of granulation, ash content, moisture, acidity and amylolitic activity was equalized. Production of hard biscuits was carried out under industrial conditions according to a standard recipe. Physical parameters and chemical properties of the biscuit samples were measured. The sensory analysis of the samples was carried out, too. Statistical analysis of the results obtained has shown that the quantity and quality of wet gluten had no effect on length and width of biscuit samples. Differences among the samples were statistically significant however in terms of height and weight. The minimum deviation in dimensions and weight of the biscuits comparing to manufacturing specifications were noted in samples produced from flour with 22% of wet gluten. According to the results achieved, this content of wet gluten is optimal for production of hard biscuits.
Key words: *hard biscuits, wet gluten, rheological properties, physical and chemical parameters*

INTRODUCTION

Technology of flour-confectionary products is based on technological quality properties of flour as a basic raw material. When combined with other materials in a mechanical process, flour has the ability to produce dough of various consistencies. From the aspect of a product recipe, good quality flour implies the ability of flour to absorb higher sugar content and develop strong structural consistency without affecting the hardness of the product (Faridi et al. 2000).
Despite all the efforts to create different types of biscuits with higher nutritional values - e.g. biscuits with an addition of buckwheat flour (Shin, 2007; Šimurina et al. 2009; Filipičev et al. 2011), or use of soy product (Nikolić and Joka, 2003; Oluwamukomi et al. 2011) - the consumption of a standard hard biscuits from wheat flour is still widespread. Production of biscuits and related products in B&H over the past years has increased and in the period from 2007 to 2009 this increase was about 20% (SG/LJ, 2010).
Hard biscuits have certain properties that make them different from other baked products. They have provided geometric shape, slightly rounded sides, a flat, smooth and shiny upper surface with furrows and regular shape holes. Structure of the cross-

section is layered with tiny oval-spread shape and uniformly distributed pores. When chewing, the hard biscuit is crisp, firm, dry, and it softens slowly and it does not stick to the palate. During chewing, the biscuit softens and releases the aroma, flavor and taste (Gavrilović, 2003). Dimensions of hard biscuits are an important factor of quality when considering the packing and the net weight label. If the biscuits spread too much, it cannot be put in the box without breaking. If the spread is too little the box is not completely filled and the net weight is not correct (Hoseney, 1994).
Although the selection and production of soft wheat varieties intended for the production of biscuits is the focus of many studies (Labuscragne et al. 1996; Pajin et al. 2005), the quality of flour for production of hard biscuits is not always satisfactory or uniform. This is particularly reflected in the protein content. It is recommended that the flour with lower wet gluten (WG) content and lower sedimentation value be used for production of hard biscuits.
According to literature and production practice, WG content in flour intended for the production of hard biscuits allows a range of 20-24% and 9.5 to 11% of total proteins. However, even this, relatively narrow range of wet gluten content may lead to variations in quality, particularly in terms of width/ length ratio of the biscuits.
Given the importance of this problem in everyday practice, the aim of this paper was to investigate the optimum amount of WG content in the flour range of 20 to 24% in order to provide for the best quality hard biscuits.

MATERIAL AND METHODS

In this research we used samples of flour T-500 of uniform quality, except for WG content. The flour was purchased in the mill "Dukat", Tešanj, harvest 2009. The content of WG in the flour samples was 20%, 22% and 24%.
Production of hard biscuits was carried out under industrial conditions (Agrokomerc, Velika Kladuša), according to a standard recipe and the process known to the manufacturer. The experiment included the production of hard biscuits out of three samples with different content of WG (20%, 22% and 24%). The production was carried out in 10 replications, i.e. from each sample a total of 10 mixing were made.

Analysis of flour samples

Physical, chemical, farinograph (water absorption, dough development, stability, softening degree, quality number and quality group) and extensograph (area under curve, extensibility, resistance, max resistance and R/E) tests and amylolitic activity of flour samples and balancing thereof were carried out according to Kaluđerski and Filipović (1990) to determine the appropriateness of flour for hard biscuits production: water content (%), ash content (%dm), flour granulation, sifting the samples on the lab sieves according to Büchler (share of individual fractions is expressed in %), acidity according to Shulerd method, wet gluten content (%) was determined by manual washing, a total protein content N X 5,7 (%dm) was determined by Kjeldahl method, sedimentation by Zeleny, fat content by Soxhlet (%dm) and rheological analyses. Sample testing was performed in parallels, taking into account the allowable deviations between the measurements defined for each method. The results show mean values of the tested parameters.

Analysis of hard biscuit samples

To determine physical and chemical properties of hard biscuits it was analyzed the following (Trajković et al. 1983): moisture content in biscuit samples (%), ash content (%), crude protein content N x 5,7 (%) by Kjeldahl method (macro-method), fat content (%) by extraction of fat with an undetermined quantity of fresh solvent and acid pre-treatment of sample according to Weibl-Stoldt. Energy value of the final

product was calculated using the conversion factors for energy values.[1] Chemical parameters were determined on an average sample taken from each replication, i.e. from each mixing (10) in triplicates. The results show mean values.
Measuring the size of biscuit samples (length, width and height) was carried out using an electronic digital caliper (MIB Germany). There were 30 parallel measurements in 10 replications (N=10X30). Sensory evaluation of 10 hard biscuits samples was carried out according to Gavrilović's method (2003). The evaluation was performed by a panel of 5 skilled participants. Evaluation was carried out on an average sample taken from each individual mixing, from each replication. The results show mean values of the five evaluators for each individual parameter in all ten (10) replication.
Statistical processing included one-factorial analysis of variance (ANOVA-Microsoft Excel 2007). Determined differences were tested with Tukey test held at 0.05.

RESULTS AND DISCUSSION

Flour 1 had the lowest, while Flour 3 had the highest content of WG and proteins. Flour containing WG in the specified range is used in biscuits industry. The protein content suitable for producing hard biscuit ranges from 9.5 to 11% (Đaković, 1997). Observing this parameter, it can be concluded that sample 1 has insufficient flour protein content for the production of hard biscuits (Tab. 1).

Table 1. Physical and chemical properties of flour samples quality

	Flour 1	**Flour 2**	**Flour 3**
Moisture (%)	13,80	14,00	14,10
Ash content (%dm)	0,50	0.51	0,52
Wet gluten (%)	20,00	22,00	24,00
Fat content (%dm)	0.70	0.60	0.50
Proteins N x 5,7 (%dm)	8,00	9.50	11,00
Acidity	1,80	1,90	1,80
Sedimentation	25,00	27,00	29,00
Water absorption (%)	55.30	57,00	58,00
Dough development (min)	1,50	1.75	1,25
Stability (min)	1,50	1,50	2,75
Softening degree (BU)	80,00	70,00	60,00
Quality number	59,00	66,40	65,40
Quality group	B1	B1	B1
Area under curve (cm^2)	62,0	68,5	88,3
Extensibility (mm)	127,0	148,0	168,0
Resistance (BU)	340,0	360,0	360,0
Max resistance (BU)	380,0	443,0	470,0
R/E	2,7	2,4	2,1
Max viscosity (AU)	368	375	367

Sedimentation of tested samples ranged from 25 in samples 1 to 29 in sample 3. The higher the gluten content, the higher the sedimentation value. According to Bešlagić (2005) the sedimentation of flour for production of hard biscuits should be in the range of 25-30, so all flour samples are adequate for hard biscuits production according sedimentation value. Based on farionograpf measurements, all the samples belong to the quality group B1 (Tab. 1). Based on planimetric areas under

[1] Official Gazette of BiH No. 85/08

the extensograpf curve (62.0 to 88.3 cm2), extensibility (127-168 mm) and resistance (380-470 BU), the samples are classified as so-called "weak flours". According to Kovačević (1991) samples 2 and 3 would be more appropriate for hard biscuits production. Flour for biscuits should have a maximum value of viscosity of 300-400 AU (Manley, 2000). The values of this particular parameter in tested samples are within specified range. Based on overall analysis of flour samples it can be seen that the general quality of the samples is balanced in all quality parameters, except for content and quality of wet gluten, which again is in accordance with the set aim.

Table 2. Particle size distribution

Particle size (μm)	Share (%)		
	Flour 1	**Flour 2**	**Flour 3**
<37	12,0	12,4	12,6
37-55	9,0	9,2	9,3
55-75	19,9	20,0	20,5
75-120	30,5	30,8	31,0
120-150	26,7	25,7	24,7
150-190	1,4	1,3	1,5
190-250	0,3	0,4	0,2

More than 50% of flour particles are in the range up to 120 μm, and more than 20% of particles are in the range of 120-150 μm which, according to Gavrilović (2003) and Spasojević (2006) can be considered as optimal value for the production of biscuits (Tab. 2). Based on the classification of flour by particle size distribution by Gavrilović (2003), the examined samples are classified into the category of “weak sharply flour”

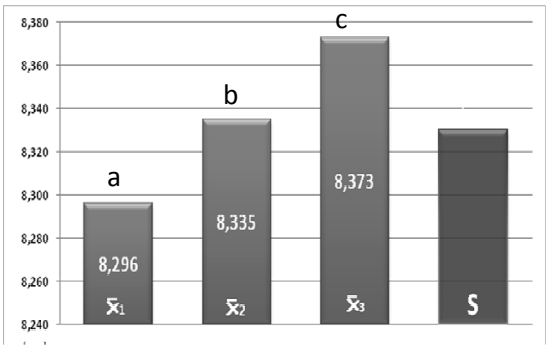
Table 3. Chemical content of biscuit samples and energy values (N=30)

	Hard biscuit sample 1	**Hard biscuit sample 2**	**Hard biscuit sample 3**
Moisture (%)	2,5	2,8	3,1
Fat (%)	13,5	13,0	12,3
Protein (%)	5,5	6,8	7,5
Ash content (%)	0,52	0,53	0,55
Carbohydrates (calculating %)	78,0	76,9	76,5
Energy value (KJ/100g)	1919,0	1903,9	1883,1

Higher content of WG in the flour ensures higher water content in the biscuits under the same baking conditions (Manley, 2000).

Based on one-factorial analysis of variance it was established that the amount of WG made no significant effect on the dimensions of the hard biscuits samples: width and length. However, in terms of height and weight of samples the effect of WG was statistically significant. Testing of differences has shown that the height and weight were the greatest in samples with WG of 24% (Fig. 1 and 4).

When compared to the values from manufacturing specification, there are differences in terms of height of biscuit samples – height of sample 1 is lower by 1.033 mm, while the height of sample 3 by 1.038 mm higher than defined values. The closest to the defined height value was sample 2, where mean deviation from the defined value was 0.025 mm. In practice, this deviation is considered insignificant, while deviations in samples 1 and 3 are considered significant. Given the height deviations, these samples would cause problems in the packing phase. Biscuits from sample 1, being of a lower height, would add to the weight of the packing as the package would include more pieces. In contrast, smaller number of biscuits from sample 3 could be fit into the package, which would lead to a lower weight than declared on the label.



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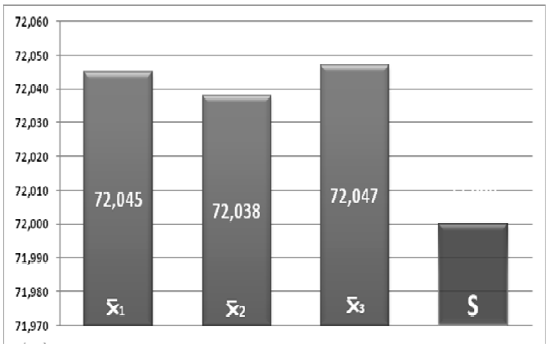

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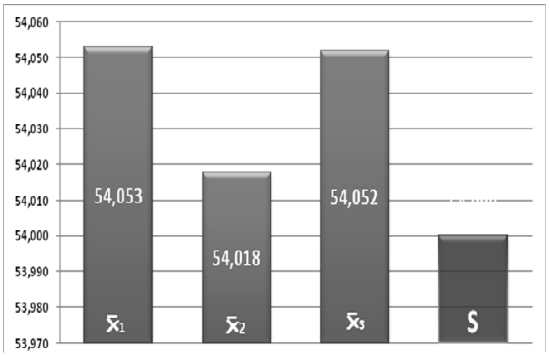

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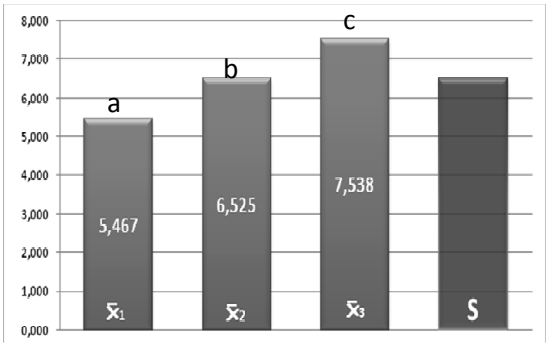

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Fig. 1-4. Means of mass (g) 1, length (mm) 2, width (mm) 3 and height (mm) 4 of hard biscuit samples (N=300) (S – values as per manufacturing specification)
Different letters mark significant difference between means (P<0.05) by Tukey test

Table 4. Sensory properties of biscuit samples

Properties	Scores		
	Hard biscuit sample 1	**Hard biscuit sample 2**	**Hard biscuit sample 3**
Shape	1,5	1,5	1,5
Upper surface	2,0	2,5	2,0
Lower surface	1,6	2,0	1,6
Fracture	2,0	2,0	2,0
Structure of fracture	2,4	3,0	2,4
Chewing	4,0	4,0	4,0
Odor, taste	5,6	5,0	5,0
Total evaluation	18,5	20,0	18,5

Biscuit samples made out of three different kinds of flour were rated descriptively as excellent. Sample 2, which was made of flour with 22.0% of WG scored maximum points, while samples 1 (with 20.0% WG content) and 3 (24.0% WG content) scored 18.5 points (Tab. 4). Of these three samples, the flour with 22.0% WG content proved to have the best sensory properties.

Differences in sensory properties of the samples were manifested in upper and lower surface and structure of the biscuits. Samples 1 and 3 scored lower points for these properties.

All samples scored maximum number of points for aroma and flavor. Accordingly, we can assume that quantity and quality of gluten in flour ranging between 20.0 – 24.0% do not affect the taste and aroma. This may be a partial confirmation of the quality of flour recommended for production of hard biscuits, although both increased or decreased level of gluten may cause practical problems in the packing phase. One could expect that improperly packed products will lose their favorable sensory properties due to cracking or breaking.

CONCLUSIONS

Based on these results, the best quality hard biscuits are made of flour with 22,0% of wet gluten and this can be considered the optimal value for production of hard biscuits, as it provides a uniform dimensions of final product, especially in terms of height, which is little or no different from the manufacturing specifications and therefore does not cause any delays and problems in packing phase. Using the flour with this gluten content enables uniform quality of hard biscuits, and corrections of the recipe or technological parameters are minimal.

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COPPER CONTENT IN KOMBUCHA FERMENTED MILK PRODUCTS

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ABSTRACT: Copper belongs to a group of essential elements, and needs to be replenished through food in certain quantities every day. But on the other hand, ingestion of large amounts of copper has an adverse effect on the human body, so that food should be subject of control in terms of copper content. In this work the copper content was determined in twelve different samples of kombucha fermented milk products using potentiometric stripping analysis, which was preceded by the determination of optimum conditions for carrying out the analysis. Fermentation liquid used as inoculum for the fermentation of milk is obtained by cultivation of kombucha on cooled tea. Four different kinds of tea were used for preparing tea: winter savory, peppermint, stinging nettle or wild thyme. Thus, four different kinds of fermentation liquids were obtained. The technique of potentiometric stripping analysis is applied with the dissolved oxygen as an oxidant, and the mercury film electrode as a working electrode. The copper content in samples was calculated using standard addition method. The contents of copper in the analysed kombucha fermented milk products were within the range from 0.016 mg/l to 0.036 mg/l, with a mean value of 0.25 mg/l. On the basis of the results of this study we can conclude that daily consumption of kombucha fermented milk products provides significant amount of copper to the human body in relation to milk, that is necessary for adults and children alike.
Key words: *copper, kombucha, fermented milk products, potentiometric stripping analysis*.

INTRODUCTION

Cooper belongs to the essential elements that are necessary for normal functioning of the human body. Copper is an integral cofactor for numerous enzymes that play key roles in various cell processes including oxidative metabolism, neurotransmitter synthesis, free radical detoxification, iron uptake and production of connective tissues (Copper in human health, 2012). Daily copper requirements for adults are 1.5-3 mg, and for children 1-2 mg (Švarc-Gajić, 2009). Copper deficit can cause thyroid problems, arthritis, and fragile bones (Švarc-Gajić, 2009). On the other hand, copper falls into the group of heavy metals. Ingestion of large amounts of copper can affect cardiovascular, gastrointestinal and nervous system. Due to its oxidant potential, copper can cause undesirable changes in processed food, related to browning reactions, lipid oxidation and consequently loss in nutritive value (Ferreira, 2005). Therefore, it is very important to monitor its content in food. Milk and fermented dairy products are very important human nutrients. They are known as an excellent source of calcium, magnesium and zinc, and provide small amount of iron and copper (Jigam et al., 2011). Consumption of milk and fermented dairy products is associated with beneficial health effects, beyond their pure nutritional value. Several reports indicated that dairy products could serve as carrier for other functional ingredients, such as phytosterols, fatty acids and various kinds of probiotic bacteria (Mattila-Sandholm et al., 2002).

Kombucha is a traditional fermented tea beverage that has gained popularity in the United States, as it is increasingly associated with health-promoting effects (Greenwalt et al., 1997). Kombucha was historically consumed in China, Russia and Germany, but today, it is a popular fermented tea beverage worldwide. Kombucha

colony is composed of two types of symbiotic microorganisms: yeasts and acetic acid bacteria (Vitas et al., 2011). Since kombucha is tea fungus, most often it is cultivated on black or green tea. After about 7-10 days incubation at room temperature, the mat, that consists of kombucha colonies, is transferred into a new fermentation, and the final product is acidic, slightly carbonated beverage, comprised of sugars, organic acids, tea components, vitamins and minerals, resembling cider (Greenwalt et al., 1997). The positive effects of these substances on the human body have long been known, and recent studies are described in the literature (Dufrense and Farnworth, 2000).
The objective of this study was to evaluate the optimal conditions for the performance of potentiometric stripping analysis (PSA), and to determine the copper level in kombucha fermented milk products, obtained by the addition of inoculums of kombucha cultivated on extracts prepared from winter savory, wild thyme and stinging nettle by potentiometric stripping analysis

MATERIAL AND METHODS

Samples

Samples of kombucha fermented milk products were obtained from the Laboratory of Analytical Chemistry at the Faculty of Technology Novi Sad. Fermentation liquid used as inoculums for the fermentation of milk is obtained by cultivation of kombucha on cooled tea. Tea was prepared by adding 70 g sucrose and 2.25 g of tea in 1 l of boiling tap water. Four different kinds of tea were used for preparing tea: winter savory, peppermint, stinging nettle or wild thyme. Thus, four different kinds of fermentation liquids were obtained. The prepared tea was cooled to room temperature, strained and then 100 cm^3 of inoculum from a previous fermentation was added. A glass jar was then covered with fabric bandwith for air, and kombucha incubation was performed at room temperature, for seven days.
For the production of kombucha fermented milk products, a pasteurized, homogenized milk with 2.8 % milk fat was used, from the manufacturer "AD IMLEK", Belgrade, department "Novosadska mlekara", Novi Sad. To the milk with 2.8 % milk fat, 10 % (v/v) of kombucha inoculums was added. Fermentation was performed at 37ºC, 40ºC and 43ºC, in 17 hours of duration, until a pH value of 4.5 was reached. Coagulated milk was then cooled and homogenized by mixer.

Instrumentation

An automatic system for potentiometric and chronopotentiometric stripping analysis used in this research was designed on the Faculty of Technology, Novi Sad as a result of cooperation with company Elektrouniverzal, Leskovac. Analyses were performed in a three-electrode cell consisting of a working electrode, a reference electrode and a counter electrode. Mercury film deposit at glassy carbon disc electrode was used as a working electrode. A platinum wire (φ = 0.7 mm, l = 7 mm) served the purpose of a counter electrode, and the reference was the Ag/AgCl, KCl (3.5 mol/dm^3) electrode. Mercury film was formed on the glassy carbon surface by galvanostatic deposition from the solution containing 0.2 g/dm^3 of mercury(II) and 0.02 mol/dm^3 of hydrochloric acid with the current of -50 µA for 240 s. Prior to each mercury film formation, the surface of the glassy carbon electrode was cleaned mechanically by filter paper wetted with acetone, and then, with doubly distilled water.
The technique of PSA (potentiometric stripping analysis) consists of several steps. The first phase includes a metal ion deposition from examined solutions on the thin mercury film of a working electrode. To maximize the amount of the concentrated metal the solution is stirred during this phase. After the second, calming phase, in the third phase the deposit is dissolved with the dissolved oxygen, and during this phase

relation between potential and time was measured. Oxidation potential is a qualitative characteristic, while the oxidation time is a quantitative one. Compared with other instrumental techniques such as the neutron-activation analysis, the atomic absorption spectrometry, that can be used for determination of copper, and other metal ions, PSA is the cheapest and the quickest method for carrying out a determination (Munoz et al., 2002, Švarc-Gajić et al., 2005).

Chemicals and solutions

All chemicals used in this work were of ultra pure grade ("Suprapur" – Merck). For all dilutions and dissolutions doubly distilled water was used. All vessels and cells were washed with nitric acid (1:1), distilled and doubly distilled water. These cautions were taken to prevent contamination from impurities in chemicals and dirty laboratory glassware.

Mercury stock solution (1 g/dm^3) was prepared by dissolving elemental mercury in nitric acid by heating and diluting with doubly distilled water. Copper stock solution (8.457 g/dm^3) was prepared by dissolving copper shavings in nitric acid (1:1). Working solution of copper was prepared by diluting stock solution with doubly distilled water.

RESULTS AND DISCUSSION

Potentiometric stripping analysis for determining the copper content was conducted on twelve samples of kombucha fermented milk products and a control sample. The control sample was a pasteurized, homogenized milk, with 2.8% milk fat, used for the production of kombucha fermented milk products. Each sample was analysed in five replicates.

Determination of the optimal conditions of the PSA

For determining the optimal conditions of the PSA, the effect of the solution stirring rate, the electrolysis potential, type and electrolyte concentration and electrolysis time were examined. All experimental parameters of the PSA were examined in the matrix of the sample, and were defined on the basis of five replicates for each examined parameter value.

To define the optimal stirring rate during preconcentration step, stirring rates from 1000 r.p.m. to 6000 r.p.m. were examined in the solution for the copper content of 20 µg/dm^3, applying the electrolysis time of 180 s. The stirring rate of 6000 r.p.m. resulted in the mechanical damage of the working electrode surface. At the stirring rate of 5000 r.p.m., analytical signal was higher, but the reproducibility was much lower. For further analysis stirring rate of 4000 r.p.m. was accepted, due to the best reproducibility and satisfactory height of the copper analytical signal (Results are shown in Table 1.).

Table 1. Influence of the stirring rate at copper analytical signal

Stirring rate (r.p.m.)	Oxidation time (s)	CV (%)
1000	1.09 ± 0.43*	19.7
2000	1.82 ± 0.26	7.1
4000	2.36 ± 0.30	6.3
5000	2.93 ± 0.65	11.1

*mean value ± 2SD (n = 5)
CV – coefficient of variation

In order to achieve greater sensitivity, selectivity and sharpness of the analytical signal, it is necessary to choose an appropriate electrolysis potential. Within that, electrolysis potential was examined in the range from -1.20 V to -0.51 V (Table 2.), with electrolysis time of 180 s, and stirring rate of 4000 r.p.m. for the copper content

of 20 µg/dm^3. The best reproducibility and satisfactory height of the analytical signal was obtained at the potential of -0.91 V, and it was applied in further investigations.

Table 2. Influence of the electrolysis potential at copper analytical signal

Electrolysis potential (V)	Oxidation time (s)	CV (%)
-1.20	1.76 ± 0.14*	7.9
-1.15	1.75 ± 0.08	4.4
-1.00	1.35 ± 0.06	4.6
-0.91	1.25 ± 0.03	2.6
-0.69	1.09 ± 0.04	4.1
-0.51	1.16 ± 0.05	4.3

*X_{mean} ± 2SD
CV – coefficient of variation

Hydrochloric, sulphuric, acetic and nitric acids were investigated as types of supporting electrolytes. Given that analytical signal in hydrochloric acid was sharp and reproductive, this electrolyte was accepted for further investigations.
Influence of the hydrochloric acid concentration was investigated in the range of 0.025 - 0.3 mol/dm^3, with electrolysis time of 180 s, and stirring rate of 4000 r.p.m. for the copper content of 20 µg/dm^3. The results are shown in Table 3.

Table 3. Influence of the hydrochloric acid concentration at copper analytical signal

c_{HCl} (mol/dm^3)	Oxidation time (s)	CV (%)
0.025	2.61 ± 0.67*	13.0
0.050	1.92 ± 0.28	7.3
0.100	2.62 ± 0.35	6.8
0.150	1.74 ± 0.55	16.1
0.200	1.77 ± 0.48	13.4
0.300	1.91 ± 0.14	3.6

*X_{mean} ± 2SD
CV – coefficient of variation

The best reproducibility of the determination was obtained in the 0.3 mol/dm^3 hydrochloric acid solution (CV = 6.8 %), but the concentration of 0.1 mol/dm^3 of hydrochloric acid was accepted as optimal, due to higher analytical signal. Influence of the electrolysis time was investigated in the range from 120-480 s in the solution of the copper content of 10 µg/dm^3, 20 µg/dm^3 and in the solution without added standard. The best reproducibility of the copper analytical signal was obtained for the electrolysis time of 180 s, and it was applied in further investigations. Limit of detection (LOD) and limit of quantitation (LOQ) are determined by applying the criteria 3 SD and 10 SD, and they were 0.006 mg/l and 0.020 mg/l, respectively. The copper content in samples was calculated using standard addition method.

Determination of the copper content in samples

Electrolysis was performed at the potential of - 0.91 V, for electrolysis time of 180 s. After the electrolysis step, which was performed under conditions of vigorous stirring (4000 r. p. m.), the solution was left quiescent for 15 s. Dissolution of deposited copper was performed in the conditions of diffusive mass transfer due to the oxidation with the dissolved oxygen. The determined copper contents in samples are shown in Table 4. The presented results represent the mean value ± 2 standard deviation of five replications of each sample. Samples were coded with letters following by number. Letters indicate kind of tea used for kombucha cultivation: winter savory tea – WS, peppermint tea – P; stinging nettle tea – SN; wild thyme tea – WT; while numbers 1, 2, 3 indicated fermentation at temperature: 37℃, 40℃ and 43℃, respectively.

Table 4. Determined copper content in samples of kombucha fermented milk products (kombucha cultivated on winter savory tea – WS; peppermint tea – P; stinging nettle tea – SN; wild thyme tea – WT)

Samples of kombucha fermented milk products	Temperature of fermentation (ºC)	Copper content (mg/l)*
WS1	37	0.022 ± 0.002
P1		0.033 ± 0.001
SN1		0.036 ± 0.010
WT1		0.031 ± 0.007
WS2	40	0.022 ± 0.002
P2		0.016 ± 0.001
SN2		0.019 ± 0.003
WT2		0.021 ± 0.005
WS3	43	0.023 ± 0.005
P3		0.028 ± 0.006
SN3		0.027 ± 0.002
WT3		0.025 ± 0.005
Milk		0.015 ± 0.003

**mean value ± 2SD (n = 5)*

The contents of copper in the analysed kombucha fermented milk products were within the range from 0.016 mg/l to 0.036 mg/l, with the mean value of 0.025 mg/l. The mean value of determined copper content in milk was 0.015 mg/l.
As can be seen from Table 4., kombucha fermented milk products contain higher concentrations of copper than milk. Given that samples were obtained in laboratory, increased copper content in samples most probably originate from the raw materials that were used in their preparation. According to the data from previous studies, kombucha beverage also contains certain amount of copper which is about 0.010 mg/l (Petrović et al., 1999). There are wide variations in the published data in terms of the copper concentrations of different kinds of tea, but there are no precise data about the copper content in the types of tea that were used for sample preparation.
Milk contains limited amount of copper. Foods especially rich in copper include bean, sea food products, cereal, vegetable and meat. (Zheng et al., 2007). Results showed that kombucha fermented milk products are richer source of copper compared to milk, so they consumption provide a higher amount of copper.

CONCLUSIONS

In this work copper content was determined in twelve different samples of kombucha fermented milk products using potentiometric stripping analysis, which was preceded by the determination of optimum conditions for carrying out the analysis. Obtained results showed that kombucha fermented milk products are richer source of copper compared to milk. Daily consumption of kombucha fermented milk products provides a certain amount of copper to human body, that is necessary for adults and children alike.

ACKNOWLEDGEMENTS

This investigation is financially supported by the Ministry of Education and Science of the Republic of Serbia (Grant III 46009).

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THE CHANGE IN MICROBIOLOGICAL PROFILE DUE TO THE OSMOTIC DEHYDRATION OF PORK MEAT

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ABSTRACT: The aim of this study was to investigate effects of osmotic dehydration on microbiological profile of pork meat, in order to indicate the usefulness of this drying treatment. Samples of pork meat were dehydrated in sugar beet molasses and aqueous solution of sodium chloride and sucrose (AOS), at three different temperatures (20^0C, 35^0C and 50^0C). Also, a_w values of the fresh and processed meat were determined and the change of the microbiological profile between the fresh and dehydrated meat was examined. It was detected that the process of osmotic dehydration does not cause deterioration of the initial microbiological profile of the pork meat, but rather improve it due to increasing dry matter content and lowering a_w value of the processed meat. In all processed meat samples, decreased microbial load, was noticed. Better results of the reduction of the present microorganisms in pork meat were obtained in the process of osmotic dehydration of meat in molasses at the higher temperatures.
Key words: *Osmotic dehydration, pork meat, sugar beet molasses, microbiological profile*

INTRODUCTION

Meat is a very perishable foodstuff due to the high water content, so it represents an ideal environment for the growth of microorganisms, particularly bacteria (Modi, 2009). Microbial contamination of the meat is dependent on the microbial load of the host organism and the hygiene practices employed during slaughter, processing and distribution (Nollet and Toldrá, 2011).
Traditional drying process have been very successful in slowing the rate of microbial spoilage and extending the shelf life of meats (Toldrá, 2010). Preservation methods like drying, canning and freezing have been applied to prolong the shelf life of foods, but these methods produce food products that are low in quality compared to their original fresh state (Ratti, 2009). Compared to the other preservation treatments osmotic dehydration has a noticeable advantages providing shelf-stable and quality processed products, furthermore is environmentally acceptable and energy efficient process. (Abbas El-Aouar et al., 2006).
Osmotic dehydration (OD) is a process of the partial removal of water before further drying by direct contact of foods (fruit, vegetable, meat and fish) with a suitable hypertonic solution. Driving force for water removal is the concentration gradient between the surrounding solution and the intracellular fluid (Lević at al., 2008). Mass transfer process is caused by a difference in osmotic pressure and simultaneously three transfer process take place: water withdrawal from product to solution, solute diffusion from solution into the product, and leaching out of the products own solutes (Koprivica et al., 2010).
Concentrated sucrose solution, sodium chloride solutions and their combinations are usually used as hypertonic solution (Mišljenović at al., 2009). Sugar beet molasses emerges as an appropriate medium for osmotic dehydration. The application of sugar

beet molasses as osmotic agent has many advantages: it is nutritionally richer than sucrose and NaCl, sensory acceptable, always accessible and cheap raw material (Koprivica et al., 2008). High content of solids (around 80%) provide high osmotic pressure in the solution and allows greater loss of water during OD and enhances the efficiency of this process. On the other hand, specific chemical composition of molasses enriches chemical and nutritional composition of dehydrated products (Lević et al., 2005).
The objective of this research was to examine the influence of two different osmotic solutions at three different temperatures on the microbiological profile of the fresh pork meat after the process of OD as a confirmation of the process safety.

MATERIAL AND METHODS

Fresh pork meat from the shoulder blade, for the experiment was purchased on the local butcher shop in Novi Sad, shortly before use. Prior the treatment all working areas and tools were thoroughly washed, cleaned and disinfected with the pharmaceutical ethanol 70% vol. Fresh meat was cut into cubes of dimensions approximately 1x1x1cm. Aqueous osmotic solution (AOS) of sodium chloride and sucrose was prepared by mixing three components, commercial sugar in the quantity of 1200 g/kg water, NaCl in the quantity of 350 g/kg water and distilled water. The second osmotic solution was sugar beet molasses from the sugar factory in Pećinci, Serbia. The material to solution ratio of 1:5 was used in all experiments. The experiments were carried out under atmospheric pressure at the temperature of the process of 20^0C, 35^0C and 50^0C. The process was performed in laboratory jars. Samples of meat were dipped into AOS and sugar beet molasses, and the immersion lasted for 5 hours. On every 15 minutes meat samples in osmotic solutions were agitated to provide better homogenization of the osmotic solutions. After osmotic dehydration meat samples were washed with sterilised water and gently blotted with paper towels to remove excessive water from the surface.
Analysis of variance (ANOVA) was performed using StatSoft Statistica, for Windows, ver. 10 program.
Water activity (a_w) of the osmotically dehydrated samples was measured using a water activity measurement device (TESTO 650, Germany) with an accuracy of ±0.001 at 25^0C.
Determination of the *Salmonella* spp., *Escherichia coli*, **the** total number of bacteria and *Enterobacteriaceae* was done by the SRPS EN ISO 6579 ; SRPS ISO 16649-2 ; SRPS EN ISO 4833 and the SRPS ISO 21528-2, respectively.

RESULTS AND DISCUSSION

In the table 1. data of the average a_w values and standard deviation of the fresh and dehydrated meat in AOS and molasses at the temperatures of the process at 20^0C and 35^0C and 50^0C are shown. Fresh sample of meat in initial state had a_w of 0.940±0.01 which is close to the optimum growth level of most microorganisms (Modi, 2009). In all samples after the process of osmotic dehydration lowered a_w values were observed which should lead to the reduction of microbial load. All three samples of meat dehydrated in AOS as osmotic solution and at three different temperatures showed lower a_w values (0.843±0.03; 0.851±0.01 and 0.853±0.03 at 20^0C; 35^0C and 50^0C, respectively) in comparison to the meat dehydrated in molasses at the same temperatures (0.871±0.031; 0.887±0.021 and 0.868±0.021 at 20^0C; 35^0C and 50^0C, respectively). AOS was more effective in lowering water activities in meat regardless of the temperature. This is probably due to the higher values of solid gain in processed meat, because it is known that increased solute concentration means decreased a_w values (Vereš, 1991). Positive influence on the

reducing a_w value of the meat dehydrated in AOS was most expressed at 20^0C. When molasses was used as osmotic medium the lowest a_w value was obtained at 50^0C.

Considering that most meat spoiling bacteria do not grow below a_w value of 0.91(Vereš, 1991), achieved a_w values of the meat dehydrated in both osmotic solutions are lower than a_w values that inhibit bacterial growth. In addition, this a_w values prevents growth of the most microorganisms except moulds since the lowest water activity allowing moulds growth is 0.80 (Vereš, 1991). It may be concluded that process of osmotic dehydration ensure a_w values which are within a specified range for meat quality and safety.

Table 1. Average a_w values and standard deviation of the fresh and dehydrated meat at three temperatures

	Temperature of the OD process:		
	20^0C	35^0C	50^0C
a_w values of fresh meat	0.940 ± 0.01		
a_w values of meat dehydrated in AOS	0.843 ± 0.03	0.851 ± 0.01	0.853 ± 0.03
a_w values of meat dehydrated in molasses	0.871±0.031	0.887 ± 0.021	0.868±0.017

The results of the microbiological analysis of the fresh and dehydrated meat are presented in table 2. Pathogenic bacteria such as *Salmonella* spp. which are frequent contaminants of meat, in the neither of the analyzed samples were found.

The number of *Escherichia coli* is an important indicator of process hygiene. There was no observed presence of *Escherichia coli* in the meat before and after osmotic dehydration in both osmotic solutions and these results are in accordance with the hygiene production criteria of the Serbian National Regulation(72/2010).

Table 2. Microbiological analysis of the fresh and dehydrated meat at different temperatures

Microbiological analysis for:	Fresh meat cut in laboratory	Temperature of the OD process:	Dehydrated meat in AOS	Dehydrated meat in molasses
Salmonella spp. (negative/10 g)	0 ± 0^a	20^0C	0 ± 0^a	0 ± 0^a
		35^0C	0 ± 0^a	0 ± 0^a
		50^0C	0 ± 0^a	0 ± 0^a
Escherichia coli (CFU/g)	0 ± 0^a	20^0C	0 ± 0^a	0 ± 0^a
		35^0C	0 ± 0^a	0 ± 0^a
		50^0C	0 ± 0^a	0 ± 0^a
Total number of bacteria (CFU/g)	$(4.33\pm0.31)E+05^a$	20^0C	$(2.17\pm0.76)E+05^b$	$(1.17\pm0.29)E+05^b$
		35^0C	$(3.57\pm0.51)E+04^c$	$(3.10\pm0.10)E+04^c$
		50^0C	$(2.17\pm0.12)E+04^c$	$(1.50\pm0.26)E+04^c$
Enterobacteriaceae (CFU/g)	$(1.93\pm0.25)E+02^a$	20^0C	$(2.00\pm1.00)E+02^a$	$(1.67\pm1.15)E+02^a$
		35^0C	0 ± 0^b	0 ± 0^b
		50^0C	0 ± 0^b	0 ± 0^b

[abc] different letters in the superscript in the same row indicate significant statistical difference between the values, at level of significance $p<0.05$

Total number of bacteria in the fresh meat cut and prepared for the osmotic dehydration in laboratory was (4.33±0.31)E+05 CFU/g, table 2.

ANOVA has showed that there was significant statistical difference between the total number of bacteria in dehydrated samples and the fresh meat, in all cases, irrespective of the temperatures of dehydration and used osmotic solutions. This proves that the process of OD has a significant influence on reducing the total number of bacteria in the osmotically treated meat. The reductions of the total

number of bacteria in dehydrated samples in comparison to the initial total number of bacteria in the fresh meat were 50%, 91.77%, 95% for samples dehydrated in AOS at 20^0C, 35^0C and 50^0C respectively, and 73.08% , 92.85%, 96.54% for samples dehydrated in molasses at 20^0C, 35^0C and 50^0C respectively. These data point out that higher temperatures of the process positively contribute to higher reduction of bacterial load. Additionally, meat dehydrated in molasses has higher lowering of the total number of bacteria at the corresponding temperatures in comparison to the meat dehydrated in AOS. The most obvious reduction of bacterial load was at the highest temperature that was used in meat dehydrated in molasses, from initial (4.33±0.31)E+05 CFU/g to (1.50±0.26)E+04 CFU/g.

ANOVA has showed that there was no significant statistical difference between the total number of bacteria of the meat dehydrated in AOS at 20^0C and the meat dehydrated in molasses at 20^0C. Also there was no significant statistical difference between the total number of bacteria of the meat dehydrated in AOS at 35^0C and 50^0C and the meat dehydrated in molasses at 35^0C and 50^0C, but there was significant statistical difference in the total number of bacteria in meat dehydrated in the same osmotic solution but at the different temperatures. This indicates that temperature has a significant influence, while the nature of the osmotic solution does not have a significant influence on the reduction of total number of bacteria of the osmotically dehydrated meat.

Serbian National Regulation (72/2010) doesn't determine reference values for the total number of bacteria for the meat pieces, but tracking the change of this parameter can indicate the level of hygiene of the process and the sustainability of the produced semi product. The results of the reduction of total number of bacteria in any dehydrated meat in comparison to the fresh meat indicate that the proces of OD is hygienically safe.

Number of *Enterobacteriaceae* in the fresh meat was 193±25 CFU/g (table 2). After the process of OD number of *Enterobacteriaceae* in the dehydrated meat has lowered in samples dehydrated in both osmotic solutions at the all temperatures, except for the sample dehydrated in AOS at the 20^0C in which negligible increase of the number of *Enterobacteriaceae* was observed (3.5%). The reductions of the number of *Enterobacteriaceae* in dehydrated samples in comparison to the initial number of *Enterobacteriaceae* in the fresh meat were complete (100%) for samples dehydrated in both solutions at the higher temperatures of the process (35^0C and 50^0C), and 13.47% for sample dehydrated in molasses at the 20^0C.

ANOVA has showed that there was no significant statistical difference between the number of *Enterobacteriaceae* of the meat dehydrated either in AOS or in molasses at 20^0C and the fresh meat. Also there was no significant statistical difference between the number of *Enterobacteriaceae* of the meat dehydrated in AOS at 35^0C and 50^0C and the meat dehydrated in molasses at 35^0C and 50^0C, but there was significant statistical difference in the number of *Enterobacteriaceae* in meat dehydrated in the same osmotic solution but at the different temperatures. This indicates that temperature has a significant influence, while the nature of the osmotic solution does not have a significant influence on the reduction of the number of *Enterobacteriaceae* of the osmotically dehydrated meat. Data point out that higher temperatures of the process more effectively reduce the number of *Enterobacteriaceae*, and also the molasses has more sucess in it.

Although, Serbian National Regulation (72/2010) doesn't determine reference values for the total number of *Enterobacteriaceae* for the meat pieces, on the basis of the change of this parameter can be concluded that the level of hygiene of the process and the sustainability of the produced semi product is high enough and that the process of OD is hygienically safe.

CONCLUSIONS

Achieved a_w values of dehydrated meat indicate that growth of microorganisms in that environment would be inhibited. On the basis of measured a_w values can be assumed that OD of meat contribute to reduction of microbial load, providing products extended sustainability. The results of the microbiological analysis of the fresh and dehydrated pork confirmed the validity of this assumption.
Better reduction of the present microorganisms in pork meat were obtained in the process of OD of meat in molasses in comparison to the OD of meat in the AOS at the corresponding temperatures.
Higher temperatures contributed to higher reduction of the present microorganisms in fresh pork meat. The best results in reducing the number of the microorganisms was achieved after OD of meat in molasses at 50^0C.
OD of the fresh pork meat in both osmotic solutions at the temperature of 22℃ has given the satisfactory microbiological parameters which are in accordance with the requirements of Serbian National Regulation (72/2010). At this temperature, due to the absence of the need for extra input of energy, OD represents energy efficient process, which provides safe products.
The microbiological profile of the osmotically dehydrated meat has shown that the OD is hygienically safe process and semi products are microbiologically stable for further technological production.

ACKNOWLEDGEMENTS

These results are part of project supported by the Ministry of Education and Science of the Republic of Serbia, TR-31055, and 2011-2014.

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INFLUENCE OF HIGH INTENSITY ULTRASOUND AS DRYING PRETREATMENT ON THE TEXTURE PROPERTIES OF PEARS

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ABSTRACT: High intensity ultrasound as novel food processing technology is promising in the field of drying of food products. Pretreatment of foodstuff using ultrasonics can significantly shorten drying time, which potentially have economical (electricity and time) and nutritional (lower drying temperatures, less impact on nutritional content) benefits. Cavitation produced by ultrasound increase number of pores and pore size which effectively impacts diffusion speed of water from core to the surface of fruits. However, changes in fruit matrix can have negative impact on textural properties of fruit. Main goal of this research is investigation of influence of ultrasonic pretreatment on drying time and texture of pears, and finding optimal processing parameters for drying. Experiments include pretreatment of pear samples using 20 to 100 % of maximal ultrasonic power (400 W) for 4 minutes. All samples were dried in the infrared dryer at 60 ^{o}C. Using instrumental texture analyzer samples were cut and hardness and elasticity were calculated based on obtained graphs. It is evident that ultrasound had significant impact on shortening of drying time (from 175 to 80 min). However this also mean that at the 80 % and 100 % of maximal power there are significant changes in textural properties, mainly in hardness and elasticity. Optimal amplitude was 60 %, which shortened drying time to 110 min, while retaining most of the hardness and elasticity compared to original untreated fruit.
Key words: *ultrasound, drying, pear, texture analysis*

INTRODUCTION

One of the main reasons for removal of moisture from pears and production of pear chips is longer shelf life, as lack of water inhibits growth of microorganisms and enzyme activity. Traditional sun drying of fruits has inherent hygienic and other problems, such as dependence on weather (Guiné, 2008). That process is also relatively expensive, and nowadays other methods of drying are commonly used. Infrared drying is one of methods which are effective for drying of thin slices. As well as conventional air drying, infrared drying is energy intensive, which can lead to high production cost (Fernandes et al, 2008). High drying temperatures required for removal of water from fruit damages nutritional characteristic of dried fruits, as well as flavor and colour (Velić et al, 2004.). Lowering of temperature significantly prolongs drying time which also negatively impacts fruit characteristics. Longer drying time also means higher costs for electricity and employees, and such method is not always economically viable (Brncic et al, 2010.).
High intensity ultrasound is effective method for shortening of drying time of various fruits (Fuente-Blanco et al, 2006). Cavitation bubbles which explosively collapse can produce microscopic channels (pores) in the fruit matrix, which influence internal and external transport of water from fruit (Carcel et al, 2007; Azoubel et al, 2010.). Besides shorter drying times which lead to significantly lower energy consumption, this also allows using lower temperatures. This in turn ensures higher vitamin and antioxidant content in the final product compared to standard drying processes. Ultrasonic cavitation which enlarge existing and create new pores in fruit tissue matrix could also negatively impact sensory properties, and lead to changes in

textural properties such as hardness and elasticity. Thus determining of optimal ultrasound and drying parameters is crucial to ensure consumer acceptance of final products and minimizing drying times.

MATERIALS AND METHODS

Pears (*p. communis* var. Williams, obtained from local market) were peeled and 5x5x0.5 mm slices were cut out. 15 g of pear slices were immersed in 100 mL of distilled water and treated with 24 kHz ultrasound (UP400s, Hielscher, Germany) with maximal power of 400 W. Each batch of samples was treated with amplitudes of 20, 40, 60, 80 and 100 % of full power, using full cycle during 4 minutes. After treatment samples were dried in infrared dryer (LJ16, Mettler-Toledo,UK) at 60 ^{o}C, until moisture content drops to 10 %. Instrumental analysis of texture was performed using TA.HDPlus (Stable Micro Systems, UK), and hardness and elasticity were calculated based on obtained TPA curves. Test was performed using blade set, with speed of 1 mm/s, until full cut through sample is evident. All samples were analyzed using 500x optical microscope. Statistical analysis of obtained data was performed using Statistica software package (Statsoft, USA).

RESULTS AND DISCUSSION

Drying time gradually drops with the increase of ultrasonic intensity, from 175 min for untreated samples to 80 min for samples treated with 100 % amplitude, as shown in figure 1. Using Tukey post hoc test it is determined that there are significant differences between drying times using various amplitudes, and $p_{100}>p_{80}>p_{60}>p_{40}>p_{20}$. It is evident that drying time is significantly shortened using ultrasound pretreatment, with minimal drying time achieved by using full power. This can be explained by influence of cavitation bubbles on cell walls and structure matrix of pear. Increase in pore dimension and creation of new microscopic channels allows for faster internal transport of water to the surface. Such enhancing of water diffusivity can shorten drying time, as presented by Azoubel et al (2010), but in turn can also have significant impact on textural properties. As shortening of drying time is less important than retaining of consumer acceptable properties, optimal ultrasonic amplitude was in the range from 20 to 60 %.

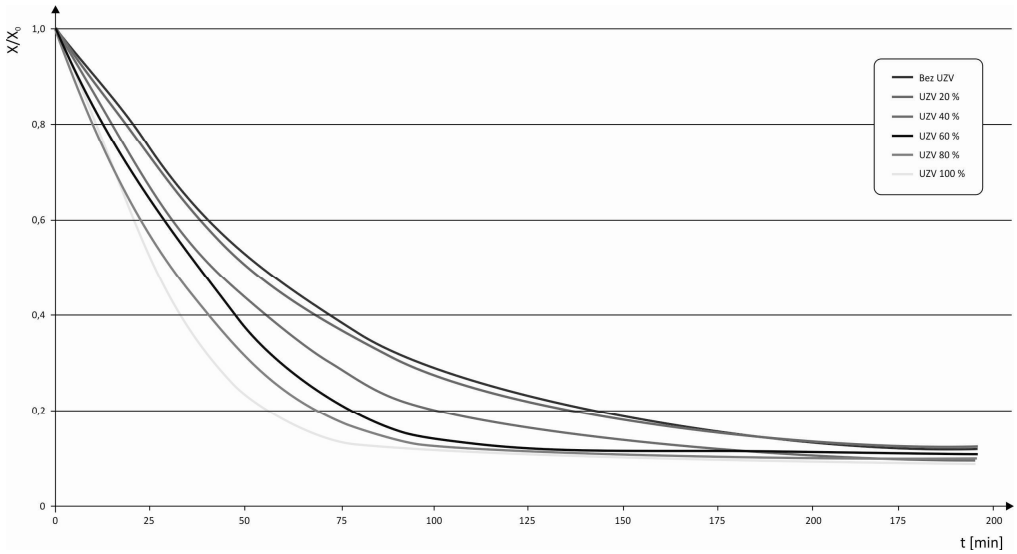

Figure 1. Drying time of untreated sample and samples treated using 2, 40, 60, 80 and 100 % of maximal ultrasonic power.

Hardness and elasticity of samples were also changing with the increases in ultrasonic intensity. This is main reason why samples treated with two of highest intensities have unacceptable sensory properties, and only those treated using amplitudes of 60 % and below appear to be acceptable from consumer perspective. Hardness, elasticity and work needed for the first bite in the sample, as shown in figures 2 and 3, increase with the increase in ultrasonic amplitude. Changes in matrix can be best seen in the figure 4. It is evident that cavitation bubbles damage cellular structure of treated samples, which confirms significant changes in textural properties.

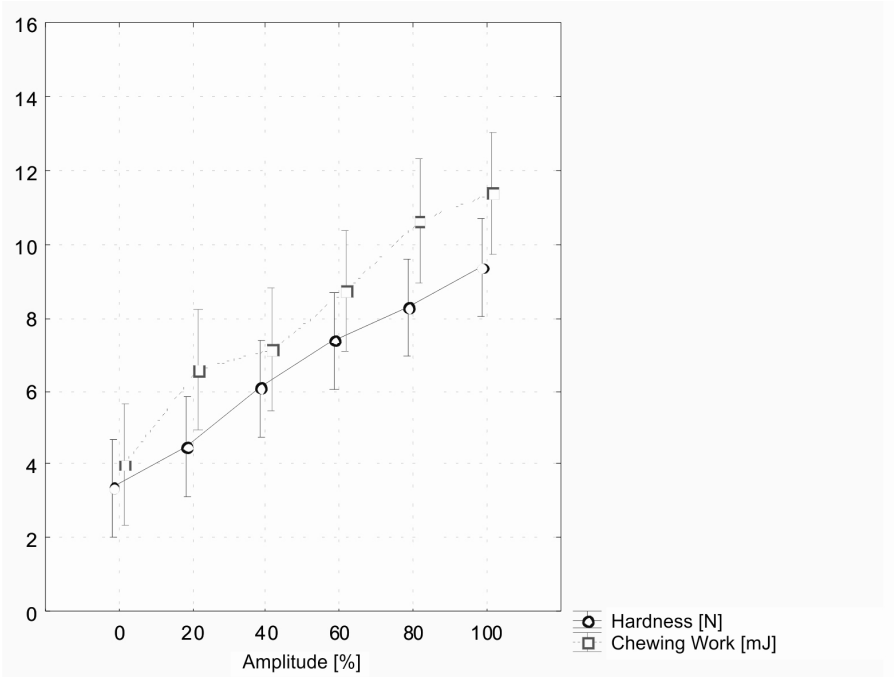

Figure 2. Influence of ultrasonic amplitude on hardness and chewing work of samples, using MANOVA method.

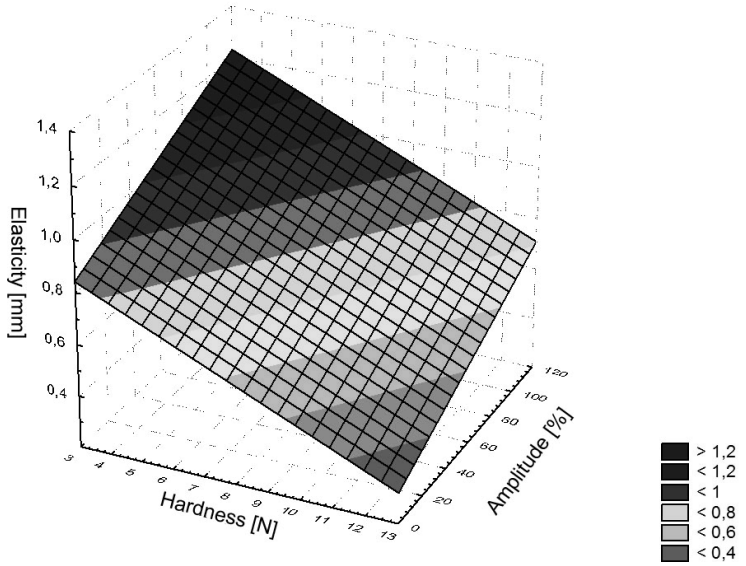

Figure 3. Influence of amplitude on elasticity and hardness of samples.

As main goal of this research is optimizing of process parameters (primarily ultrasonic amplitude) to minimize texture damage and drying time, it is evident that 100 % of amplitude leads to minimal drying time. However, as only 20, 40 and 60 % of amplitude satisfy second condition for minimizing of texture changes, optimal

ultrasound parameters for drying of fruits are 60 % of maximal power (240 W) during 4 minutes. Further increase in processing time would lead to more damages to structure.
Based on the obtained results, ultrasonic pre-treatment can be excellent addition to the conventional drying processes such as infrared or air drying. While texture changes must be minimized, drying time is still significantly shorter compared to drying of untreated samples. With calculation of energy consumption for the ultrasonic treatment, and energy savings obtained by shorter drying times, this kind of treatment can produce significant reduction in processing costs.

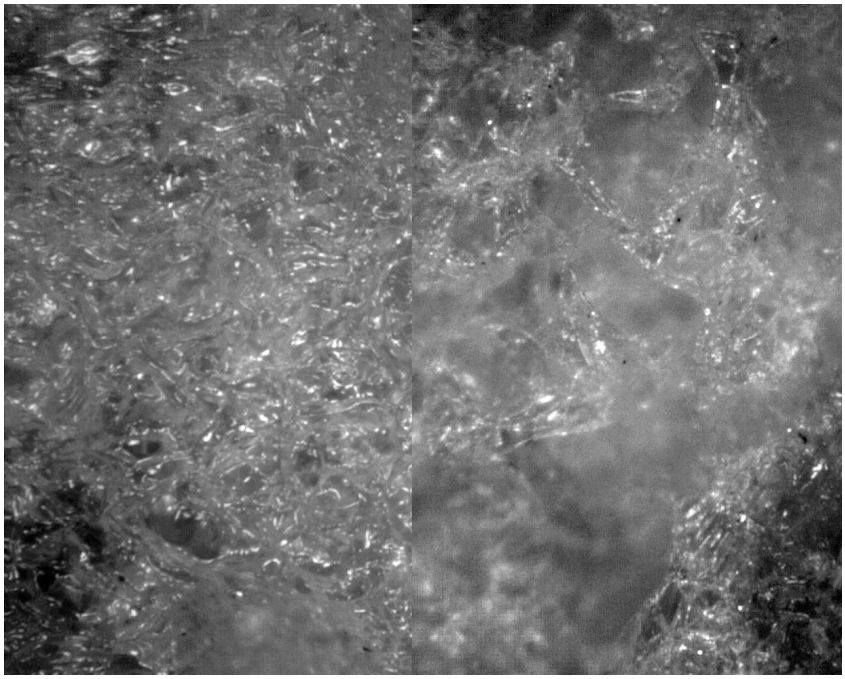

Figure 4. Side-by-side microscopic images of untreated sample, and sample treated with maximal intensity.

CONCLUSIONS

Ultrasonic pretreatment significantly shorten drying time, from 175 min (untreated) to 80 min (treated using full power). All amplitudes tested had influence on drying time, while maximal power consequently had maximal influence. At the same time, maximal power had largest influence on the hardness, elasticity and chewing work of samples. Optimal ultrasonic intensity was 60 Wcm^{-1} during 4 minutes, as this substantially shortens drying time, while retaining changes in the measured textural parameters in acceptable range. Loss of hardness and elasticity at larger intensities was too large, and despite shortest drying times, such samples were deemed unacceptable from consumer perspective. This research shows that ultrasonic pre-treatment is viable option for enhancing of drying process, as there is possibility for time and energy savings, as well as retaining of nutritional quality of raw fruits.

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PARTICLE SIZE DETERMINATION IN FOOD PRODUCTION: APPLICATION TO CRYSTALLIZATION PROCESS IN SUGAR BEET PROCESSING

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ABSTRACT

ABSTRACT: Particles size has important role as parameter for control and evaluation of food processing and quality characteristic of final product. In sugar cane or sugar beet processing the most important influences of particle size are evident in phase of juice purification and crystallization. Particle size of calcium carbonate has great effect to the nonsucrose compounds removal from raw juice and filterability of juices in the juice purification. Crystallization is the final stage in production of organic or nonorganic sugar.
The paper has dealt with applications of Coulter Counter technique in evaluation of crystallisation process in technical sucrose solutions under laboratory conditions. The crystal growth rate was determined by measuring crystal size and crystal size distribution. The Rosin-Rammler-Sperling-Bennet function was applied for evaluation of characteristic size and uniformity of crystals.
In sugar industry determination of crystal size distribution is applied in order to optimize production of crystal footing, evaporation and cooling crystallisation. Crystal size distribution has a significant influence on the processes of centrifugation, drying, sifting, storing and on the quality characteristics of the final product. The aim of crystallization is to produce a maximum amount of crystal sugar, sucrose crystals of best possible quality without any twin crystals, conglomerates or very fine grain and reduce energy consumption.
Key words: *nonsucrose compounds, sucrose crystallization, crystal size distribution, growrh rate dispersion*

INTRODUCTION

Sugar industry is one of the largest industries in the world, and crystallization is an important stage which determines the yield and quality of the sugar from sugar beet and sugar cane. White sugar, produced in modern sugar factory, easily meets the quality criteria according to the EU sugar market regime. Some industrial sugar consumers introduce additional requirements as content of insoluble matter, turbidity, creating floc, crystal mean size and size distribution, shape of crystals etc. (Clarke, 1995). Sugar is primarily a food, but it is also included in the formulation of many pharmaceuticals.
The kinetics of the growth of sucrose crystals usually represents reaction occuring on the surface of the different crystal faces and at the crystal-solution interface. Supersaturation is the driving force of the crystallization. It is known that minor changes in the supersaturation, temperature, the conditions of seeding, the hydrodynamic conditions, the amount of the crystals and purity of technical sucrose solution can have significant impact on the crystal growth kinetics. Nonsucroses can affect to the sucrose solubility, growth kinetics, morphology and quality of the crystals. The presence of nonsucroses inside the crystal lattice has important effect

on the sugar quality especially as colour, ash and water content. The various faces of sucrose crystal have different growth rates and the most rapid faces include mother liquor more rapidly than the others.
Two kinds of methods have application for measuring sucrose crystal growth rate: single and polycrystals (Hook, 1984). Single method is convenient and rapid but suffers from a variability of individual crystals growth (Bubnik et al.,1992; Sgualdino et al., 2005). Polycrystalline method is based on the measuring the size distribution of a crystals crop and can be adapted to simulate actual production process (Gros et al., 2001; Grbić, 2009).
The current work was targeted towards understanding sucrose cooling crystallization from technical solutions, which composition are similar to those in industrial crystallization of sugar beet processing.

MATERIAL AND METHODS

Growth rates of sucrose crystals were measured in presence of nonsucrose compounds, using $2.6 \cdot 10^{-3}$ m^3 batch cooling crystallizer at constant agitation speed. Concentration of nonsucrose compounds was increased by mixing different amounts of sugar beet molasses with beet white sugar and pure water. Technical sucrose solutions were kept for one hour undersaturated at 80 ℃ before cooling. Saturation state and point of seeding were calculated according to dry substance and sucrose content (Šušić et al., 1995). In analogy to the practice in industry, fixed amount of slurry was intoduced in the point of seeding. It was used in the amount of 1.0 % w/w. Slurry was prepared by wet-milling a sugar/isopropanol suspension in a ball mill. The experiments continued by lowering the solution temperature, following defined programme during the period of 3 hours. The supersaturation was reached by decreasing sucrose solubility.
The analyses of solutions were carried out according to the methods published in the Laboratory Handbook (Milić et al., 1992). The dry substance was measured by a digital automatic refractometer (ATR-ST plus, Schmidt + Haensch, Germany). The sucrose content was determined polarimetrically by a digital automatic saccharometer (Saccharomat IV, Schmidt + Haensch, Germany).
Crystal size distributions in slurry and solutions were obtained by off-line automated technique (Coulter Counter, model ZM, England). Coluter Counter determined number or volume distribution by direct crystal counting based on electrically conductivity measurement (Grbić et al., 2007). For evaluation of characteristic size and uniformity of crystals, the Rosin-Rammler-Sperling-Bennet (RRSB) function was applied (ICUMSA, 2003).

The crystal growth rate was determined as mass growth rate:

$$R_G = dm / A \cdot dt \quad (1)$$

Where is: R_G – mass growth rate, m – crystal mass (mg), A – crystal surface area (m^2), t – time (min).

RESULTS AND DISCUSSION

Parameters of technical sucrose solutions are presented in Table 1. The purity values in range from 90.55 to 92.30 % were based on the characteristics of the thick juice. Concentration of nonsucrose compounds ranged from 7.70 to 9.45 g/100DS. The points of solutions seeding showed the metastable zone of supersaturation.

Table 1. Parameters of technical sucrose solutions quality

Parameters	Experiments			
	A	B	C	D
Dry substance (°Bx)	80.40	80.00	80.40	80.00
Purity (%)	90.55	91.72	92.08	92.30
Nonsucrose compounds (g/100DS)	9.45	8.27	7.91	7.70
Seeding temperature (°C)	67	70	69	69
Seeding supersaturation	1.20	1.14	1.18	1.16
Final temperature (°C)	27	30	28	31

Figure 1 presents particles size distribution of slurry which was applied for the initial crystals formation. Diameters were divided in the intervals and the number of particles was counted in every interval (dual treshold technique). Mean particles size d_{RRSB} was 20 µm and uniformity coefficient 4.2.

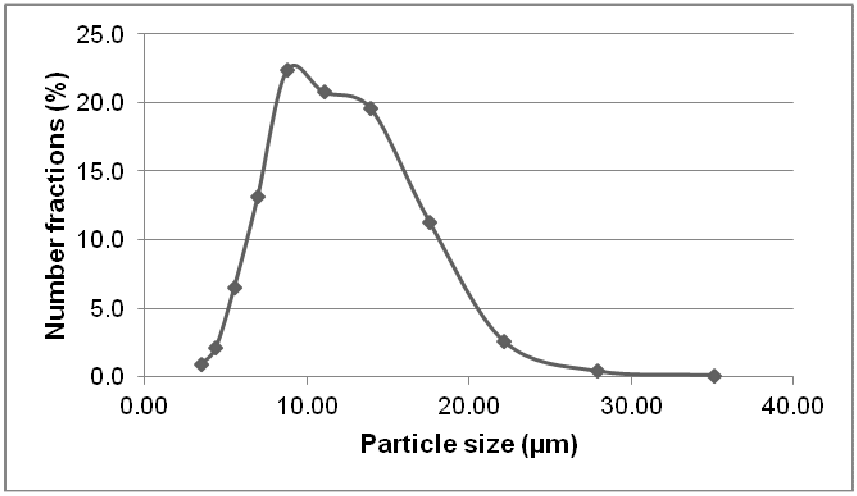

Figure 1**.** Histogram for number distribution of particles size in slurry

The changes of sucrose crystal mass growth rate and mean size d_{RRSB} as function of solutions purity are present in Figure 2.

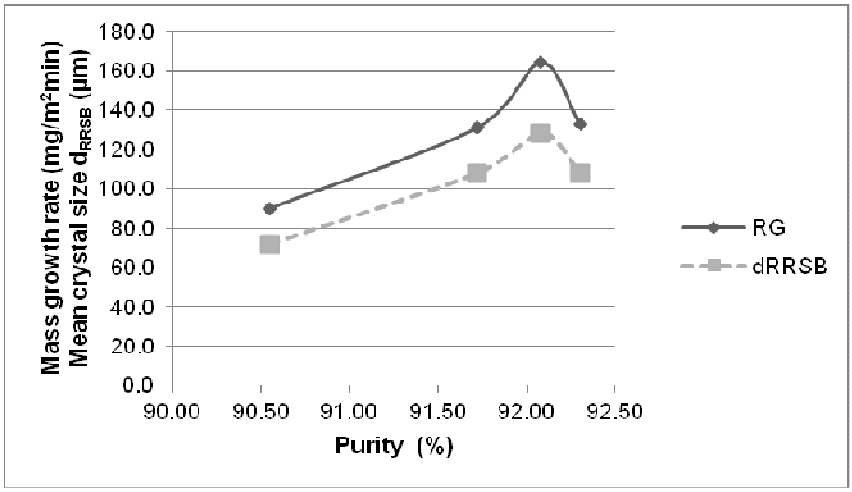

Figure 2. Sucrose crystal mass growth rate and mean size as function of solutions purity

The highest values were reached with solution purity of 92.08 %. Further increasing of purity decreased mass growth rate and mean size in the same conditions of experiments, because of secondary nucleation. In case of secondary nucleation a part of sucrose excess from supersaturation solution is also used for formation and growth of new nuclei and only the rest of sucrose excess is incorporated into the

crystal lattice originating from the slurry. Mechanism of the nonsucrose compounds influence on crystal growth rate is complex and should be understood as a result of their impact on sucrose solubility, diffusion of sucrose in solution and on surface reaction (Sgualdino et al., 1996).
The crystal size distribution is determined by number and mass distribution, mean crystal size, uniformity coefficient, spread of crystal sizes. In the crystallizer, the crystal size distribution is formed by a complex series of strongly interrelated processes referred to as the mechanism of the crystal size distribution formation. Studies on crystal growth dispersion, secondary nucleation and agglomeration can be made from crystal size distribution. Mass distributions of sucrose crystal size for different systems are present in Figure 3. By increasing of solutions purity from 90.55 to 92.08 % maximums of mass distributions were shifted to higher values of crystal size. The next increasing of purity over 92.08 % led to decreasing of crystal size for mass distribution maximum. Increase of distributions spread were obtained from higher purity solutions. The uniformity coefficients of crystal were very good in all experiments, and varied from 5.0 to 5.8.

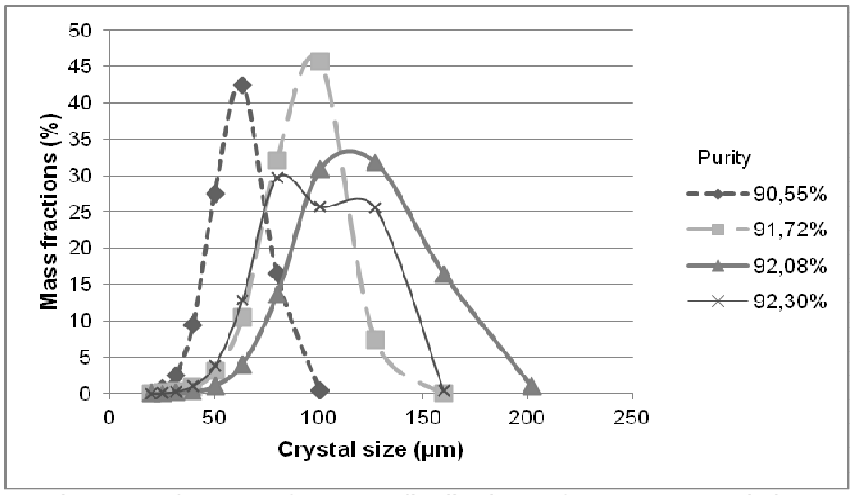

Figure 3. Histogram for mass distributions of sucrose crystal size

The change of number fraction of crystals smaller than 50 µm as function of solutions purity is presented in Table 2.

Table 2. Number fraction smaller than 50 µm in sucrose crystal mass of technical solutions

Purity (%)	90.55	91.72	92.08	92.30
Number fraction of crystal size < 50 µm	70.24	21.83	17.64	31.28

Growth rate dispersion is an important factor that influences crystal size distribution. Small crystals grow slowly than larger in the same hydrodinamic conditions, because the higher supersaturation is required for their growth. Figure 4 presents mass growth rate of particular fractions in the experiment C. The results shows that growth rates increase with crystal size (Martins and Rocha, 2007).

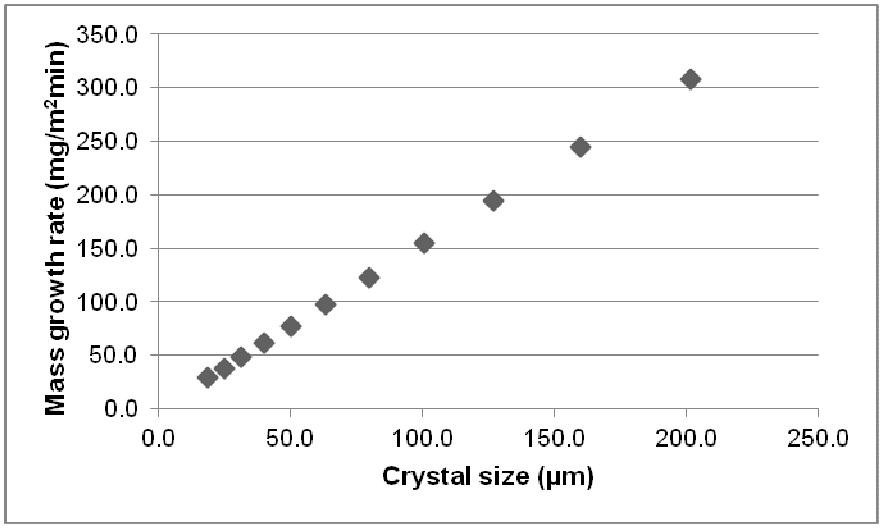

Figure 4. Mass growth rate of sucrose for different crystal sizes in the solution of 92.08 % purity

In all experiments, crystal size distributions during crystal growth satisfied the Rosin-Rammler-Sperling-Bennet (RRSB) function.

CONCLUSIONS

Based on the results of this study , the following conclusions can be made:

- Nonsucrose compounds led to reduction of growth kinetics and mean crystal size of sucrose.
- The highest values of sucrose crystal growth rate and mean size were reached with solution purity of 92.08 %. Further increasing of purity decreased mass growth rate and mean size in the same conditions of experiments, because of secondary nucleation.
- Maximums of mass distributions were shifted to higher values of crystal size by increasing of solutions purity from 90.55 to 92.08 % The next increasing of purity over 92.08 % led to decreasing of crystal size for mass distribution maximum.
- Small crystals grew slowly than larger. Dispersion of growth rate is one of the important factors that influenced the crystal size distribution.
- Uniformity coefficients from 5.0 to 5.8 presents very good values, that pointed to satisfied conditions of cooling crystallization.
- Crystal size distributions during crystal growth satisfied the Rosin-Rammler-Sperling-Bennet (RRSB) function.
- Coulter Counter technique is important tool to describe the crystal size distribution during crystallization and can contribute to a better understanding of the process.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from the Ministry of Education and Science of the Republic of Serbia (Project III 46005, 2011-2014).

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CHANGES IN NUTRITIVE QUALITY OF PORK MEAT OSMOTIC DEHIDRATION IN SUGAR BEET MOLASSES AND AQUEOUS SOLUTION OF SODIUM CHLORIDE, SUCROSE AND SUGAR BEET MOLASSES

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ABSTRACT: Osmotic dehydration is a water removal process involving soaking food in a hypertonic solution. Process of osmotic dehydration is typically used as a pre-treatment for many processes in order to improve the sensory and functional properties of food, without a significant decrease in nutritive values of the product. This paper analyzes the effects of different hypertonic solutions, after the process of osmotic dehydration of pork meat under atmospheric pressure at room temperature of 22^{o}C, to determine the nutritive quality of the final product. As osmotic agents sugar beet molasses and aqueous solution of sodium chloride and sucrose and sugar beet molasses in the ratio 1:1, were used. The ratio of raw material and hypertonic solution was 1:5, and immersion time was set to 5 hours. Fundamental chemical composition was determined by *SRPS ISO* methods, and mineral content was determined using AAS method. The results indicate that during the process of dehydration significant changes occur in water loss and growth dry matter content. The initial dry matter content of meat was 26.21% and after the immersion in molasses increases to 68.52%, while in aqueous solution of sodium chloride and sucrose and sugar beet molasses in the ratio 1:1 increases to 63.70%. Also, after the process of dehydration meat was enriched in some minerals Na, K, Ca and Fe, while the content of Mg was slightly reduced. Based on the results, it can be concluded that the applied solutions significantly improve quality of semi-products and that osmotic dehydration represent a suitable pre-treatment for meat drying. Water loss was slightly higher when sugar beet molasses was used as osmotic agent. Using sugar beet molasses as an alternative medium for the process of osmotic dehydration opens new opportunities for innovation in dehydrated products.
Key words: *osmotic dehydration, pork meat, osmotic solution, molasses*

INTRODUCTION

Osmotic dehydration is a useful process for obtaining products with moderate moisture content, which has been in recent years used in the preparation of different types of meat product. In the case of meat, water removal is combined with mild salting and liquid smoking to effectively replace long and costly salting/smoking operations (Mitrakas et al., 2008).It is frequently used as a pre-tretman (preparation) for many processes to improve sensory and functional properties of food without significantly altering the nutritional composition of the product (Beuchat, 1996). According to Rastogi et al., osmotic dehydration is becoming popular as pre-treatment in the processing of foods specifically because of the advantages of energy savings (occurs without a change in physical state of water) (Tortoe et al., 2007).
As hypertonic solutions commonly are used combined solutions of sodium chloride and sucrose with different concentration levels and ratios, and driving force for the water removal process is concentration gradient between the solution and the fluid inside the cell. During the osmotic dehydration kinetics of water removal depends

primarily on the type of osmotic agent, its concentration and temperature of osmotic solution, and the size of the matrix (Khan et al., 2011).
Osmotic dehydration is the most intense at the beginning of the process, as a result of the major difference in osmotic pressure between the osmotic solution and cell fluid of dehydrated material and a small mass transfer resistance (Salvatori et al., 1999; Mazza, 1983). Traditionally, meat was considered a highly nutritious food (Higgs, 2000). Meat is an important source of high biologically valued protein, macro and micro elements, vitamins B and vitamin D (Lombardi et al., 2005).
Treatment of soaking meat in a concentrated solution of salt/sugar provides great dehydration of product, allowing the elimination of the operation of salting and dehydration, which is commonly practiced in traditional meat processing, limiting the immersion of high salt content in muscle tissue, which causes a high salt content in the final product. Moreover, the presence of another solution besides salt, such as sucrose and/or sugar beet molasses maintained a high potential for achieving high dry matter content, or significant loss of water. At the same time large quantities of salt impregnation in muscle tissue, are interfered by the presence of sucrose. Sucrose has the “barrier effect”, forming a layer on the periphery of the product which is caused by difference in the transport of water and solutes in meat. The barrier effect is stronger if a high molecular weight sugar is being used. (Santchurn et al., 2007).
The aim of this study was to investigate the possibilities and effects of concentrated solution sodium chloride/sucrose/molasses and pure sugar beet molasses as two hypertonic solutions for osmotic dehydration on the nutritional value of fresh pork.

MATERIAL AND METHODS

For the experiment, fresh pork meat from shoulder blade, without skin, bones, fat or connective tissue, was purchased at local market 48 hours after slaughter. For the preparation of osmotic solutions were used commercially available sodium chloride and sucrose, and sugar beet molasses from sugar factory Bač.
The meat, from which the remains of fat and connective tissue were removed, was cut into cubes, dimension of nearly 1x1x1cm, using a sharp knife. After grinding, the samples were immersed in hypertonic solution R_1 (sugar beet molasses) and R_2 solution (NaCl + sucrose+ sugar beet molasses in the ratio 1:1).
The ratio of raw material and hypertonic solution was 1:5, and the immersion time of 5 hours. Osmotic dehydration was carried out at atmospheric pressure and temperature of 22℃. After the treatment, samples were taken out from osmotic solutions to be lightly washed with water and gently blotted with absorbent paper to remove excessive water.
The basic chemical composition of fresh and dehydrated meat was determined by examining:
-Moisture content (dry matter*)* (SRPS ISO 1442, 1997),
-Protein-nitrogen content (TruSpec CHN(S), LECO, metod AOAC 992.15.)
-Free fat content (JUS ISO 1443, 1997)
-Chloride content (SRPS ISO 1841-1,1999).
-Total phosphorus content (SRPS:ISO 1443,1997)
-Sugar content by Luff-Schoorl-u (Baras et al.,1983)
-Metal content (AAS- Manuals of Food Quality Control, 2. Additives Contaminents Techniques. FAO Food and Nutrition Paper, Rome, 1980.)

RESULTS AND DISCUSSION

Table 1 provides an overview of the general chemical composition of sugar beet molasses and sodium chloride, sucrose and sugar beet molasses solutions, which

were used in this experiment. The results of the basic chemical composition of fresh pork meat from shoulder blade are shown in Table 2.

Table 1. Chemical characteristics of sugar beet molasses (R_1) and the mixture solution (sodium chloride, sucrose and sugar beet molasses (R_2)

Characteristics	Sugar beet molasses- R_1	mixture solution R_2
Dry matter, %	85,04±0,13	76,24±0,10
Sucrose, %	52,32±0,42	32,22±0,16
Invert Sugar, %	0,55±0,44	3,9±0,17
Protein,%	10,38±0,06	5,66±0,01
NaCl, %	0,01±0,00	13,00±0,11
K, mg/100g	2211±52,61	2081±84,26
Na, mg/100g	1178±20,70	3704±97,53
Ca, mg/100g	211±52,71	123±6.92
Mg, mg/100g	26±15,56	13±1,91
Fe, mg/100g	10±0,94	2,2±0,07

Results are given as mean ± standard deviation (n = 3)

Table 2. Chemical composition of pork meat from shoulder blade

Characteristics	Pork shoulder
Dry matter, %	26,21±0,09
Total Protein, %	20,92±0,33
Fat, %	5,11±0,30
Phosphates (P_2O_5), g/kg	4,70±0,21
NaCl, %	0,12±0,02
Invert sugar, %	0,23±0,00
K, mg/100g	234±41,55
Na, mg/100g	54±2,35
Ca, mg/100g	7±0,37
Mg, mg/100g	28±2,35
Fe, mg/100g	1,3±0,17

Results are given as mean ± standard deviation (n = 3)

Table 3 shows the changes of chemical composition in samples of dehydrated pork meat after immersion in two different hypertonic solutions under the defined conditions.

Table 3. Change of chemical composition in pork meat after osmotic dehydration M_1, M_2 in solutions R_1 and R_2

Characteristics	M_1-pork meat shoulder after osmotic dehydration in R_1	M_2- pork meat shoulder after osmotic dehydration in R_2
Dray matter, %	68,52±4,87	63,70±0,19
Total Protein, %	36,60 ±0,34	32,60±0,19
Fat, %	7,85±0,11	6,68±0,10
Phosphates (P_2O_5), g/kg	4,44±0,31	3,80±0,00
NaCl, %	0,82 ±0,05	3,57±0,06
Sucrose, %	6,68±0,27	7,81±0,11
Invert sugar, %	7,04±0,28	8,23±0,11
K, mg/100g	1307±82,30	816±54,34
Na, mg/100g	282±53,44	544±86,88
Ca, mg/100g	36±2,52	113±0,11
Mg, mg/100g	24±1,75	13±0,63
Fe, mg/100g	3,9±0,72	2,9±0,08

Results are given as mean ± standard deviation (n = 3)

Based on the results of tables 2 and 3, the value of dry matter in dehydrated meat is increased, indicating that there was a diffusion of sucrose and other nutrients from

the solution R_1 and R_2 in to the flesh. The ratio of water loss and solid gain is an indicator of osmotic dehydration efficiency. The increase in dry matter content of fresh meat from 26.21% after dehydration was two and a half times in the meat samples M_1 (68.52%) dehydrated in molasses. Smaller increase in dry matter content (63.70%) was obtained in the meat samples dehydrated in osmotic solution R_2. Since the water loss is affected linearly by the temperature of osmotic solution, working temperature was set to 22℃, which pursued the redu ced viscosity of solution, primarily sugar beet molasses and facilitating the penetration of substances into the muscle tissue. Molasses is a poly component system and has a high density; thereby the diffusion of molasses components in muscle tissue is more difficult at lower temperatures.

By increasing the dry matter content an increase in the share of total protein and fat occurred in dehydrated meat. Protein content in meat M_1 reached 36.60% and 32.60% in M2, which helped to preserve the most important nutritional component of the meat. As for sugar, dehydrated meat has a higher content of both sucrose and invert sugar comparing fresh meat, regardless of the type of solution for dehydration. The increase in sugar content was moderate and ranged from 6.68% to 7.81% for sucrose and from 7.04% to 8.23% for invert in dehydrated meat M_1 and M_2. The amount of NaCl in meat after osmotic dehydration in R_1 was 3.57%. Since sucrose is a compound that is normally present in animal tissue, the permeability of sucrose in the muscle cell may be lower compared to NaCl (Vivanco et al., 2004). Rapid formation of a concentrated sugar solution layer on the surface of meat as well as faster impregnation salt than sugar can be explained by a lower molecular weight of salt and greater potential for mass transfer (Santchurn et al., 2007).

Sugar beet molasses represents a significant source of many minerals, especially potassium, calcium, iron and magnesium. Particularly significant is the fact that all the mineral components in the molasses are in dissolved state and the potassium is in much greater quantities than all other cations with a share of about 75% (Šušić et al., 1989). Osmotic dehydration of pork meat M_1 in sugar beet molasses R_1 lead to significant increase of K content from 234mg/100g to 1307 mg/100g, while in samples M_2 dehydrated in solution (salt/sugar/molasses) R_2, content of K was 508 mg/kg. After five hours of immersion content of Na increased five times 5 in meat M_1, and ten times in meat M_2. Increase of Ca is less expressed in sample M_1 36mg/100g compared to M_2 113mg/100g. The content of Mg decreased slightly during the osmotic dehydration of meat in a solution of molasses R_1, while larger losses were obtained during immersion in R_2, where the Mg content of fresh meat from 28mg/100g decreased to13mg/100g. The increase of Fe content in final products M_1 and M_2 is about two times higher than the same quantity before dehydration, regardless of the type of hypertonic solution. This increase of Fe in dehydrated meat is very significant, if it is known that the absorption of Fe from meat is usually 15-20%, which significantly improved functional and nutritional value of meat as food (Higgs, 2000).

CONCLUSIONS

The most significant changes in water loss took place during osmotic dehydration. Rate of water loss was two and a half times in meat dehydrated in sugar beet molasses, a slightly lower in meat dehydrated in mixture solution (sodium chloride, sucrose and sugar beet molasses). Increasing dry matter increases concentration of total protein, fat, K, Na, Ca and Fe. The content of Mg decreased slightly during the osmotic dehydration of meat in both solutions.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 31055 “Osmotska dehidratacija hrane-energetski i ekološki aspekti održive proizvodnje (Osmotic dehydration of food-energy and environmental aspects of sustainable production)”, financed by the Ministry of Science and Technological Development, Republic of Serbia.

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MICROBIAL PROFILE OF DRIED PORK PRODUCED WITH LOWER SALT CONTENT

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ABSTRACT: Production of food with lower amount of common salt (sodium) is challenge for food industry. Particularly problematic is the production of dried meat with lower amount of common salt because these products are not preserved by high temperature.
In this paper is presented the microbial profile of dried pork produced with lower amount of salt during production and storage under vacuum conditions for 120 days. Total plate count is determined during processing, in the raw meat, after curing and on 14th, 21st and 28th day of production by standard method ISO 4833:2003. During storage under vacuum conditions on the 1st day, 60th day and 120th, count of halotolerant bacteria is determined using Manitol salty agar and count of lactic acid bacteria by standard method ISO 15214:1998.
Maximum total plate count was after curing, average 5.04 cfu/g and after that it is decreased up to the end of production, average 2.68 cfu/g. Average count of halotolerant bacteria was 2.19 cfu/g in the final product in superficial layer of meat and 1.41 cfu/g in inner layer of meat, but after 60 and 120 days of storage halotolerant bacteria were not presented. Lactic acid bacteria were presented only at the beginning of storage, 2.17 cfu/g in the superficial layer of meat and 1.98 cfu/g in the inner parts of dried meat. Maximum total plate count after curing is presented due to handling with meat and it is under positive influence of added salt.
Vacuum conditions had negative influence on presence of halotolerant and lactic acid bacteria which are not presented during longer storage, because of anaerobic conditions in the vacuum packing.
Key words: *microbial profile, dried pork, lower salt content*

INTRODUCTION

Common salt (NaCl) is an ingredient that has been used since ancient time for the preservation and flavouring of meat. Sodium chloride contributes functional properties in meat products. It activates proteins to increase hydration and water holding capacity; it increases the binding properties of proteins to improve the texture; it increases viscosity of meat batters, facilitating the incorporation of fat to form stable batters; and it has essential flavouring and antimicrobial effects (Terell, 1983). In the 20th century, human nutrition becomes mostly based on consumption of meat products and snacks that contain high content of sodium chloride. Even sodium chloride is GRAS substance (generally recognized as safe), excessive daily intake of sodium has linked to essential hypertension and correlated with mortality and risk of coronary heart diseases, independent of other cardiovascular risks, including hypertension (Tuomilehto et al., 2001). Large amount of sodium originated from meat products (Wirth, 1991).
Present trends in nutrition is reducing the sodium content in meat products, as reported by Ruusunen and Puolanne (2005) and Desmond (2006), can be achieved in the following way: (1) by reducing the amount of sodium chloride added (Sofos, 1983; Lilić, 2000); (2) by substituting part of NaCl with other salts (Sofos, 1983; Terell, 1983; Guàrdia et al., 2006; Lilić et al., 2008).; (3) by using flavour/aroma enhancers and masking agents (Desmond, 2006); (4) combination of mentioned procedures (Sofos, 1983; Terell, 1983); (5) adding of spice herbs and spice extracts to meat products (Lilić

and MatekaloSverak, 2007; Matekalo-Sverak et al., 2007); (6) optimisation of the physical form of salt (Angus et al., 2005); and (7) alternative process techniques (Claus and Sørheim, 2006).
The aim of this paper was to investigate microflora of dried pork produced with lower content of sodium chloride.

MATERIAL AND METHODS

The material in this paper was pork (*m. longissimus dorsi pars thoracis*) originated from white pigs, six months old, with live average weight of 100 kg. After chilling the meat was treated with following mixture: 950 g of nitrite curing salt and 50 g of sucrose, in the amount of 300 g per 10 kg of meat. Curing lasted for 7 days and after that meat was smoked for next 7 days. Process of drying and ripening lasted for 14 days. Final products were packed in PA/PE bags under vacuum conditions and stored at room temperature for 120 days.
Samples for microbial examination were taken on the first day of production (raw meat), after curing (7^{th} day of production) and on 14^{th}, 21^{st} and 28^{th} day of production (final product). In these samples were determined the total plate count by standard method ISO 4833:2003. Samples were taken also on the 60^{th} and 120^{th} days of storage under vacuum conditions in the aim of determination of number of halotolerant bacteria using Manitol salty agar and lactic acid bacteria count that determined by standard method ISO 15214:1998.
Results of microbiological examinations are presented as average count of bacteria (log cfu/g) with standard deviation.
Sensory characteristics of dried meat were evaluated by six trained assessors (SRPS ISO 6658:2002).

RESULTS AND DISCUSSION

The total plate count (aerobic mesophylic bacteria) during the production of dried pork is presented in Figure 1. Average number of aerobic mesophylic bacteria in raw meat was 3.99 ± 1.09 cfu/g in superficial layer of meat and 2.00 ± 0.57 cfu/g in inner part of meat. After curing total plate count increased up to 5.04 ± 0.86 cfu/g in superficial part and up to 2.16 ± 1.04 cfu/g in inner part of meat. Smoking had negative influence on aerobic mesophylic bacteria which average number was 3.18 ± 0.26 cfu/g in superficial and 1.92 ± 0.98 cfu/g inner part of meat. During drying and ripening the number of bacteria was also decreased up to 2.75 ± 0.19 cfu/g on 21^{st} day of production and 2.68 ± 0.30 cfu/g at the end of production in superficial layer of meat and 1.23 ± 0.87 cfu/g in inner part of meat on 21^{st} day of production, while these bacteria were not presented in inner parts of meat at the end of production. Obtained results are in accordance with results of Sinell (1977) who found 10^2-10^7 cfu/g and with results of Ockerman and Kuo (1982) which found 2.66-2.67 log cfu/g.

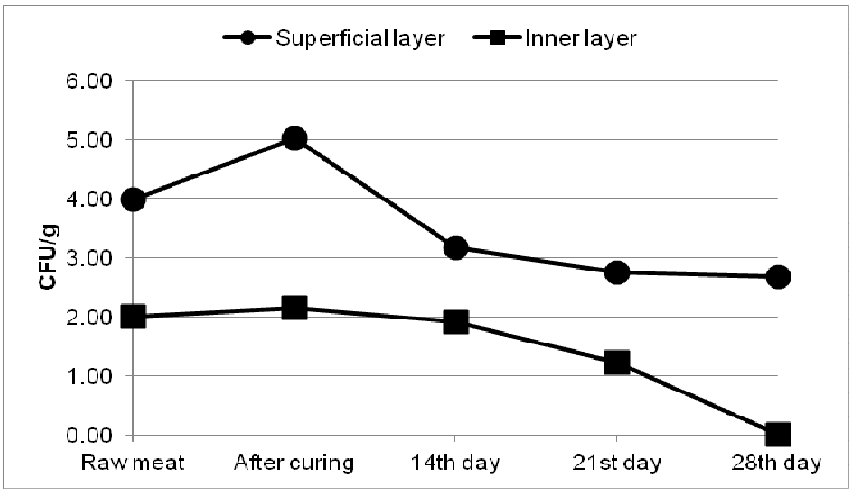

Figure 1. Total plate count in meat during production

Number of halotolerant and lactic acid bacteria is presented in Figure 2. Average number of halotolerant bacteria was 2.19 ± 0.18 cfu/g in the superficial layer of meat and 1.41 ± 1.00 cfu/g in inner part of meat, while average number of lactic acid bacteria was 2.17 ± 0.20 cfu/g in superficial layer of meat and 1.98 ± 0.33 cfu/g in inner part of meat. On 60th and 120th day of storage under vacuum conditions these bacteria were not determined. Halotolerant and lactic acid bacteria were not presented at 60th and 120th day of storage due to low amount of oxygen or total absence of oxygen under vacuum conditions. Obtained results for halotolerant bacteria are in accordance with results of Silla et al. (1990) that determined 10^2-10^6 cfu/g and with results cited by Giolitti et al. (1971) that found less than 500 cfu/g. Number of lactic acid bacteria in dried pork is 10^2-10^7 cfu/g according to results of Sinell (1977) and to results of Silla et al. (1990) that cited number of lactic acid bacteria up to 10^3 cfu/g in dry ham.

Molina et al. (1989) cited that microflora of dry ham consisted from *Pediococcus pentosaceus*, while other lactic acid bacteria are *Lactobacillus alimentarius*, *Lactobacillus curvatus* and *Lactobacillus casei* var. Rhamnosus and *Lactobacillus divergens*. Cornejo and Carracosa (1991) determined in 96% of all halotolerant bacteria belong to family *Micrococcaceae*. Both halotolerant and lactic acid bacteria have role in ripening and fermentation during the production of dried meat due to activity of lypolitic and proteolitic enzyme activity. Toldrá and Conceptión Aristoy (2010) cited that natural flora of dry ham is consisted from *L. sakei*, *L. curvatus* and *P. pentosaceus* but their number is less than 10^4 cfu/g.

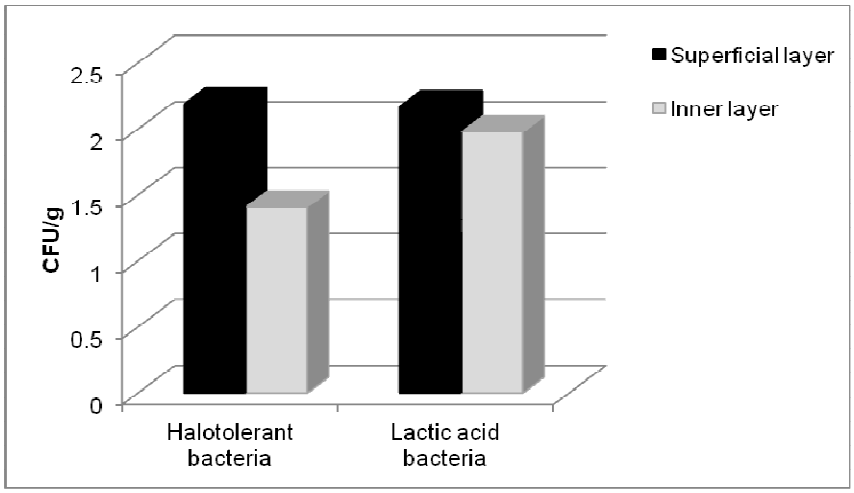

Figure 2. Halotolerant and lactic acid bacteria count in vacuum packed dried pork

CONCLUSIONS

Total plate count is higher in superficial layer of meat after curing that could be explained by meat handling during curing; but it decreased to the end of the production in both superficial and inner part, particularly in inner part it was not detectable at the end of production.
Halotolerant and lactic acid bacteria are presented in dried pork at the end of production, approximately 2 cfu/g for both groups of bacteria in the superficial layer of meat. In the inner part of meat, the number of halotolerant bacteria was average 1.41 cfu/g and number of lactic acid bacteria was 1.98 cfu/g.
Due to microaerophylic condition in the first period and anaerobic conditions in the next period of storage, halotolerant and lactic acid bacteria could not be detected in dried pork.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 31083 “Reducing of sodium content in meat products – technological posibilities, quality parametres and health aspects (Smanjivanje sadržaja natrijuma u proizvodima od mesa – tehnološke mogućnosti, karakteristike kvaliteta i zdravstveni aspekti)“ financed by the Ministry of Education and Science, Republic of Serbia.

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EFFECT OF MACERATION AND PASTEURIZATION ON COLOUR OF CLOUDY SOUR CHERRY MARASCA (*PRUNUS CERASUS* VAR. MARASCA) JUICE

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ABSTRACT: Colour is very important quality factor for fruit juices which affects consumers' preferences. Sour cherry juice colour is mainly due to the presence of anthocyanins. Fruit maceration with enzymes improves extraction of anthocyanins, while pasteurization can cause their degradation. Therefore, the aim of this study was to investigate the effect of different duration of the maceration and pasteurization on colour stability of cloudy Marasca juice. Sour cherries Marasca were used to produce cloudy juices on small scale equipment with hydraulic press.Processing included thawing (without depitting), maceration, pressing and pasteurization. Duration of maceration varyied (30-150 minutes) and juices were sampled after pressing and after pasteurization. Obtained juices were analyzed by colour measuring, total content of anthocyanins, phenols and antioxidant activity. All samples were sensory evaluated using Quantitative Descriptive Analysis. Duration of maceration and pasteurization had significant effect on colour parameters in Marasca cloudy juice. Anthocyanins were present in the highest concentrations in juices with 0.5 h of maceration. Furthermore, pasteurization affected positively on its amounts, as well as on the content of total phenols and antioxidant activity. Maceration enzymes treatment significantly affected taste attributes of Marasca cloudy juice. Maceration of 0.5h is sufficient for producing well coloured Marasca cloudy juice with good sensory attributes. Pasteurization doesn't negatively affect juice colour as well as the content anthocyanins, total phenols and antioxidant activity.
Key words: *sour cherry Marasca, juice, enzyme treatment, colour, anthocyanins, sensory evaluation*

INTRODUCTION

Sour cherry Marasca is an autochthonous Croatian cultivar, with great amount of biologically active compounds, high content of dry matter, dark red colour and intensive agreeable aroma (Pedisic *et al*., 2007, Levaj *et al*., 2010). Although Marasca sour cherry is fruit with great benefits and large number of processing possibilities, it is insufficiently used.Interestingly, sour cherry products have had an increased utilization in the food market in the past decade because of their potential health benefit (Khoo *et al.*, 2011; Kyrakosian *et al*., 2009). Belibağli and Dalgic (2007) mentioned extensively use of sour cherry juices in the food industry, as constituents of juices, jellies, marmalades, jams, wines beverages, etc. what generates a consumer market with increasing demand. Also, sour cherry juices are characterized by pronounced sweet-sour flavour which makes them very popular and preffered to other fruit juices by various customers. Marasca cherry is fruit suitable for processing into cloudy juice, containing more antioxidants and fibre than clear juices (Mieszczakowska-Frąc*et al.*, 2012). Anthocyanins are highly concentrated in the epidermal tissue of fruit and several studies conducted in recent years have shown that anthocyanins display a wide range of biological activities including antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic activities (Obón *et al.*, 2011). Process of juices separation from fruit cells in cloudy juice production is usually obtained by pressing and provides juices with nice colour, which is very important

quality factor and affects consumers preferences. Maceration of pulp with enzymes in juice production is widely used to enhance the yield of juice and to increase the contents of various compounds in the juice (Laaksonen *et al*., 2012; Pap *et al*., 2010). Its effectiveness depend on type of applied enzyme and treatment conditions (pectinolytic enzyme dose, maceration time, and reaction temperature). Most of commercial enzyme preparations contain pectinases, cellulases and hemicellulases in various ratio, which act in different way, but it is important that the enzyme doesn't degrade pectin completely, while pectin and other components of cell wall transferred to pressed juice contribute to cloudiness (Will and Dietrich, 2006; Mieszczakowska-Frąc*et al.*, 2012). To prolong shelf life of juices, pasteurization is the necessary and integral process in juice production and the thermal pasteurization is the most widely applied technique, successfully inactivating vegetative microorganisms and enzymes (Kimball, 1999). As the anthocyanins are found to be sensitive for higher processing temperature, pasteurization can cause colour degradation of sour cherry juices (Patras *et al.*, 2010). The objective of the present work was to investigate the effect of different duration of maceration and pasteurization on colour stability, chemical composition and sensory profile of cloudy sour cherry Marasca juices in order to elaborate suitable enzyme treatment to obtain juice with nice colour, health benefits and excellent sensorial characteristics.

MATERIAL AND METHODS

Juice production

Juices were produced from sour cherries Marasca grown near Zadar (Škabrnja). Fruits were harvested in June 2011, packed in polyethylene bags and kept at -18 ºC till juice production. After thawing overnight at +4 ºC and without depitting, samples were used to produce cloudy juices on small scale equipment with hydraulic press (Euclid Ltd., Croatia). Prior to pressing, fruits were warmed up in a water bath to the incubation temperature (48 ºC) and treated with Endozym Pectofruit PR (AEB group, Italy) in concentration of 40 µL/kg. Every half an hour, batch of 2 kg of sour cherries Marasca was excluded for pressing, concluding with 2.5 hour of maceration (juice code: 0.5, 1, 1.5, 2, 2.5). Control juice (0) was produced without pre-treatment. Juices were glass bottled after pressing and after pasteurization (85ºC/10 min) (juice code: 0P, 0.5P, 1P, 1.5P, 2P, 2.5P) and kept at -18 ºC until all analysis.

Methods

Soluble solids were determined by measuring the ºBrix (Atago refractometer, Japan). Total acidity (expressed as g malic acid/100 mL juice) and pH-value were determined in accordance with methods in literature (Tanner and Brunner, 1979).

Colour (CIE L*, a*, b*) analysis was performed using Konica Minolta CH-3500 D spectrophotometric colorimeter. Color of sour cherry juice was presented as L*, a*, b*, C* and H° values. C and H° values were calculat ed according to the formula $C^* = (a^{*2} + b^{*2})^{1/2}$, H° = arctan (b*/ a*) (Konica Minolta Tehnical Ins tructions, 1998).

The content of anthocyanins (TAC) in juices was determined by bisulfite bleaching method (Ough and Amerine, 1988). The molar absorption coefficient for cyanidin-3,5-diglucoside was used as a standard value. Results were expressed as mg of cyanidin-3,5-diglucoside equivalents (Cy-3,5-DG) per 1 mL of juice.Total phenols (TP) were extracted according to Coseteng and Lee (1987) and spectrofotometricaly determined using Folin-Ciocalteu colorimetric method (Ough and Amerine, 1988) and the results were expressed as mg gallic acid equivalent (GAE)/1 mL of juice.FRAP method was used for the antioxidant activity (AA) determination (Connor et al, 2002). FRAP values were calculated according to the calibration curve for trolox, and they were expressed as mmol of trolox equivalents (TE) per 1 mL of juice. All

spectrophotometric measurements were performed by UV-VIS spectrophotometer UV-Vis Unicam β.
All analyses were done in triplicate and data are presented as mean value.
The sensory evaluation (SE) of the juice samples was conducted using Quantitative Descriptive Analysis (QDA). The panel consisting of eight trained members evaluated juice samples according to the corresponding list of descriptive terms for colour (intensity), taste (sour, sweet, sour cherry taste, astringent, harmony taste, taste fullness), odour (sour cherry odour) and texture (viscosity, homogeneity). Attributes were rated on a 10-point intensity scale, in which 10 indicated the highest intensity of evaluated sensory attribute.The procedure was performed according to methods described in ISO 6564, ISO 8587, ISO 11036 and the reports of Bursać *et al.* (2007) and Bursać Kovačević *et al.* (2008) in a sensory laboratory equipped according to ISO 8589.

Statistical data analysis
All obtained results were subjected to statistical analysis using ANOVA (Statistica v. 10, Statsoft Inc, Tulsa, OK, USA) to investigate the statistical significance of maceration and pasteurization. Differences were considered significant at $p \leq 0.05$.
Principal component analysis (PCA) was applied for general assessment of similarity-dissimilarity of the evaluated sour cherry juice samples. Statistical analyses were gained by Statistica v. 10 (Statsoft Inc, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Soluble solids, pH value and total acidity values (Table 1.) of control juice (0) were in accordance with previously reported results for fresh Marasca sour cherry by Pedisic *et al.* (2007). Generally, soluble solids increased with duration of maceration (24.40 – 29.95 ^{o}Brix). Pasteurized juices mostly had higher soluble solids than unpasteurized ones. According to ANOVA ($p \leq 0.05$) it was observed that the duration of maceration significantly effected on pH value ($F=7.95$, $p=0.02002$), which decreased with longer duration of maceration (3.51 – 3.36). Although, the total acidity values (2.0 – 2.50) showed opposite trend to pH values, maceration and pasteurization had no significant effect on juices total acidity or their soluble solids. ^{o}Brix/Acid ratio in all samples was in range of 11.4 – 12.5, which was similar to results in Pedisic *et al.* (2007) report (11.8) and higher in comparison to the results of Konopacka *et al.* (2009) for untreated sour cherry sample (9.3). The highest ratio was observed in 1.5 and 2 juices and higher ratio means more acceptable juice (Fellers *et al*., 1988.).

Table 1. Physico – chemical and colour parameters of Marasca juices

Juice code	pH	Soluble solids	Total acidity	°Brix/ Acid ratio	L* (light ness)	a* (red ness)	b* (yellow ness)	C* (chroma)	H° (hue angle)
		°Brix	g malic acid/100mL						
0	3.45	25.30	2.21	11.4	2.94	20.41	5.05	21.03	13.91
0P	3.51	24.40	2.00	12.2	1.80	12.47	3.05	12.84	13.76
0,5	3.45	26.35	2.25	11.7	1.13	7.80	1.93	8.03	13.87
0,5P	3.50	27.80	2.30	12.1	0.03	0.14	0.00	0.15	15.52
1	3.42	25.80	2.25	11.5	0.67	4.67	1.14	4.81	13.75
1P	3.38	27.85	2.25	12.4	0.38	2.52	0.62	2.59	13.81
1,5	337	27.20	2.20	12.4	0.62	4.34	1.06	4.47	13.73
1,5P	3.37	27.65	2.35	11.8	0.33	2.21	0.56	2.28	14.13
2	3.37	27.50	2.20	12.5	0.50	3.46	0.83	3.56	13.48
2P	3.36	27.30	2.30	11.9	0.34	2.39	0.59	2.46	13.78
2,5	3.37	27.00	2.20	12.3	0.66	4.68	1.13	4.82	13.50
2,5P	3.36	29.95	2.50	12.0	0.26	1.81	0.45	1.87	13.88

Colour of Marasca juices were presented with parameters L*, a*, b*, C*, H°(Table 1). With increasing duration of maceration, a significantly decreasing trend in L*, a*, b* and C* values was observed in all samples. Decreased lightness (L*) in juice is mainly attributed to increased anthocyanin content as a result of enzyme treatment (Khandare *et al*., 2011), which was confirmed, in spite of falling direction of the line, with high correlation (r=0.83) between L* values and the amounts of anthocyanins in Marasca juices. The same correlation was obtained with a* and C* values. Expectedly, pasteurization caused colour degradation in obtained juices, while thermal treatment affected anthocyanins stability. Values of a* parameter, which represent the redness of Marasca juices, were almost double lower in pasteurized juices than in unpasteurized juices.

The results of total anthocyanins (TAC), total phenols (TP) and antioxidant activity (AA) of Marasca juices are shown in Figure 1. Enzyme treatment affected positively on TAC content, causing it's initially increase in all obtained juices. Among treated juices, TAC slightly decreased with duration of maceration. The highest value (2.654 mg cy-3,5-DG/mL) was obtained in sample exposed to 0.5 h of maceration. Pap *et al*. (2010) reported that enzymatic treatment resulted in the increase of anthocyanin and flavonol content of the black currant juice, what is similar to our results. According to ANOVA ($p \leq 0.05$), there were not significant effects of duration of maceration on TAC, opposite to pasteurization, which significantly affected on TAC in Marasca juices ($F=7.69$, $p=0.03924$). TAC content increased during pasteurization probably because all anthocyanins present in cloudness cells in unpasteurized juice might not react with bisulfite reagent, but after pasteurization they could be liberated in juice and could be more available for reaction. Total phenols (TP) and antioxidant activity (AA) results showed similar trend as TAC results. TP and AA amounts were the highest between 0.5 and 1.5h of maceration. Pasteurization had not caused their degradation. Correlation between TAC and AA was good (r=0.69), but there were no correlation between TP and AA. The enzyme treatment was more effective to TAC increase of Marasca juice samples than TP. The increase was about 20 - 40% in the TAC case, while the maximum TP increase was 15%.

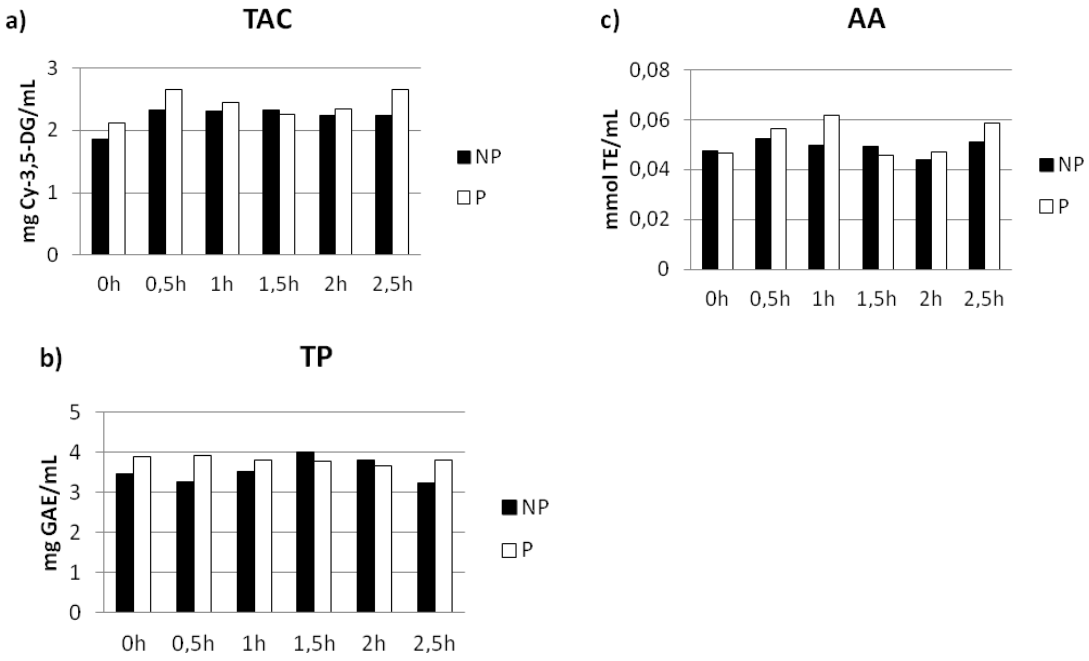

Figure 1. Total anthocyanins (TAC), total phenols (TP) and antioxidant activity (AA) of Marasca juices

Main observations of SE were that duration of maceration caused significantly increase of sweetness ($F=8.29$, $p=0.01831$) and decrease of sourness ($F=47.72$, $p=0.0.00032$) what is in accordance with increase of soluble solids ($r=0.68$) and ^{o}Brix/Acid ratio in juices (for unpasteurized juices $r=0.82$, pasteurized juices $r=0.65$). Additionaly, astringency ($F=6.30$, $p=0.03239$), and sour cherry odour ($F=13.64$, $p=0.00615$) of juices significantly decrease with duration of maceration. Pasteurization didn't negatively affect on sensory attributes, especially on colour intensity, which remained constant in all samples.

PCA was performed on all samples and variables to determine whether the different kinds of samples (sour cherry juices produced with or without different enzyme pre-treatment before and after pasteurization) had an effect on different sensory attributes. In this context, ten sensory attributes of sour cherry juice were the investigated variables.

The PCA results were two graphs (projections of variables, loading plots and cases-score plots), but the interpretation also mentioned eigenvalues of the correlation matrix, factor variable correlation (factor loadings) and case contributions, which are not discussed here.

The first two factors (PC1 and PC2) represented 64.2 % of the initial variability of the data. This was a good result, but some information might be hidden in the next factors. So, first and third factors PC3 (11.30 %) are also observed, but not shown here.

Figure 2. gives a visual representation of the differences between sour cherry juices produced with or without different enzyme pre-treatment before and after pasteurization. Regardless of pasteurization effect, sour cherry juices without enzyme treatment were positioned on the right side of the PC1 while sour cherry juices with enzyme treatment at 0.5, 1.0 and 1.5 h were positioned in the middle. Sour cherry juices with enzyme treatment at 2 and 2.5 h were positioned on the left side of the PC1, without considerable effect of pasteurization.

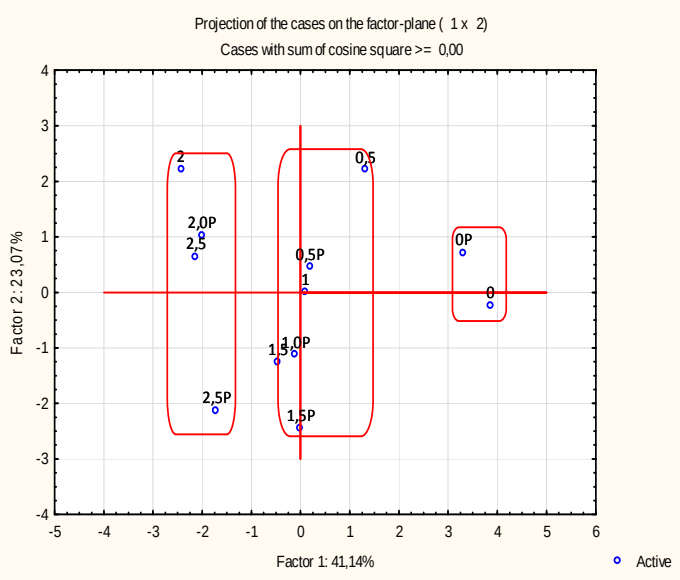

Figure 2. Principal component analysis (PC1 vs. PC2) of Marasca juices, taking into account all relevant variables. Sample labels for the score plot: P – samples with pasteurization, 0 – samples without enzyme treatment, 0.5, 1.0, 1.5, 2.0 and 2.5 – samples with duration time of maceration

Without effect of pasteurization, sour cherry juices without enzyme treatment were characterized by sensory variables such as sour taste, flavour fullness and sour cherry odour. These sensory attributes strongly correlated with the PC1. According to correlation with PC2 it can be assumed that sour cherry juices with enzyme treatment (1.5h and 2.5 h) and pasteurization are characterized by harmony taste. Due to correlation with PC3, it can be observed that sour cherry juice with enzyme treatment (0.5 h) and without pasteurization is characterized by homogeneity.

CONCLUSIONS

Presented study showed that conditions of enzyme treatment 0.5h/48 ºC prior to pressing remarkably improved the quality of sour cherry Marasca cloudy juice, resulting in enhanced anthocyanins and total phenolic content and their antioxidant activity, as well as nice colour and good sensory attributes. In addition, all mentioned components and attributes remained preserved after pasteurization (85 ^{o}C/10 min).

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EFFECT OF PROCESSING ON COMPOSITION OF NUTRITIONAL STARCHES IN PLANTAIN (*MUSA AAB*)

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ABSTRACT: Plantain, a member of the banana family is an important staple food in the tropics. It is edible when fully ripe but in most cases it is cooked before consumption. Despite its abundance in the diet of many populations, quantitative and qualitative data on various starch fractions are scarce. *In vitro* digestibility of starch for processed and unprocessed samples was determined using pancreatic α-amylase and amyloglucosidase (Englyst & Englyst, 2004), and D-glucose was measured using glucose oxidase/peroxide reagent. Resistant starch in content in uncooked samples was 36.6±1.5g/100g and 62.7±2.9g/100g for ripe and unripe samples respectively. These values were reduced in the processes samples, with figures ranging between 3.5-6.5 g/100g in the ripe samples and 15.0 - 19.9 g/100g in the unripe samples. Slowly digestible starch (SDS) values were between 0.6 and 9.2 g/100g, while rapidly digestible starch (RDS) ranged between 25.6 – 56.6 g/100g for processed samples. Starch digestibility index (SDI), which is the ratio of the rapidly released glucose to the total starch content was lowest in the grilled samples (60.0). It is concluded that processing techniques affect the type and content of nutritional starches and therefore it will be essential to optimize processing techniques to maximize the RS and SDS which are known to have positive effect on postprandial glucose response.
Keywords: Plantain, resistant starch, slowly digestible starch, rapidly digestible starch, processing

INTRODUCTION

Plantain (*Musa AAB*), a member of the banana family, serves as a major staple food in many parts of the African subcontinent. Starch is the main component of plantains, reaching up to about 80% on dry weight basis, depending on the stage of ripeness. (Da Mota *et al*, 2000; Zhang *et al*, 2005). Plantain is low in protein but relatively high in carbohydrate, minerals, and vitamins (Southgate *et al*, 1969; Best *et al*, 1984). Ripe plantain contains 66% starch and 17% sugars compared with 5% starch and 80% sugars for bananas (Ketiku, 1973). A high level of dietary fibre and resistant starch in unripe plantain and banana flours was reported by Da Mota *et al*. (2000).
Plantain is cooked in different ways before consumption in Nigeria and is believed to have therapeutic properties. The results of a recent study supports the fact that green plantain has a favorable function in the dietary management of persistent diarrhoea in hospitalized children, in relation to diarrheal duration, weight gain and costs (Alvarez-Acost *et al.,* 2009).“A plantain-based diet can be designed for diabetic people based on its low glycaemic index.”(Mohapatra *et al*, 2009). In an ileostomy study by Englyst and Cummings (1986) on the digestion and absorption of banana carbohydrates, up to 90% of the starch could be accounted for in the effluent; also, complete recovery of non-starch polysaccharides (NSP) was attained. They inferred that the amount of banana starch not hydrolyzed and absorbed from the human small intestine and therefore passing into the colon may be up to eight times more than the NSP present in the food and depends on the state of ripeness of the fruit.
Although the physiological effect of RS has been investigated in relation to reduction of the glycemic and insulinemic responses to food. (Jenkins *et al*, 1987; Asp *et al*, 1996), recent studies have indicated that there are other nutritional starch fractions

which have an impact on glycemic response. Englyst *et al*, (1992) classified starch fractions into resistant starch (RS) which are undigested starch fractions, slowly digestible starch (SDS) which are completely but slowly digested and rapidly digestible starch (RDS) which are rapidly and completely digested (table 1). Studying the impact of processing on these nutritional fractions is quite important because during processing, starch undergoes many and varied significant changes which can alter both physical and chemical properties of food as well as the food form. Starch gelatinization, pasting and retrogradation which underlie starch functionality are the three most significant phenomena in starch applications and play a very significant role in determining starch digestibility and the relative quantities of starch fractions in processed foods (Liu, 2005).
In this work we aimed at determining the proportions of various starch fractions in ripe and unripe plantains and how theses are affected by various processing methods used before they are consumed. The cooking methods (boiling, boiling with skin, grilling and frying) used in this work reflect the major ways in which plantains are consumed in a typical African diet.

MATERIALS AND METHOD

Sample collection and preparation: Cultivars of ripe and unripe plantains were purchased from Leeds city market. Unripe plantains selected were full green (stage 2) while ripe plantains used were in fully ripe stage (yellow) in colour (stage 6) on the colour index scale (Aurorea, 2009). Both ripe and unripe plantains were divided into five portions each. The peel was removed from all five portions except portion two. Portion one was and boiled, two was boiled with the skin on, three was fried while four was grilled, five was used raw. Each process was conducted three times for the four cooking methods. The samples were cut into thin slices of 2mm in diameter, freeze dried and blended into fine flour and stored way in clean plastic containers for future analysis.

Cooking processes

Boiling: 150gram of sample was boiled in 300ml distilled water for 10 minutes in a clean pot with a lid.
Grilling: 150 gram of sample was grilled at high/medium heat in a grill for 30 minutes.
Frying: 150g portion of samples were cut into slices of 2mm in diameter and deep fried in vegetable cooking oil for approximately 5 minutes.
Digestion: Total starch (TS), free glucose (FG) and the different starch fractions – RDS (rapidly digestible starch), RS (resistant starch) and RAG (rapidly available glucose) were measured in triplicate by the method of Englyst and Englyst (2004). Efficiency of starch hydrolysis and speed of water bath were first standardized using wheat flour, cornflakes and raw potato starch. Briefly, samples were first incubated with pepsin for 30 minutes at 37℃ to disrupt starch –protein interactions. The contents of the various starch fractions were measured in samples after a further incubation with pancreatin and amyloglucosidase at 37℃ in capped tubes, horizontally immersed in a shaking water bath. The incubation tubes contained glass balls for disrupting the food particles and guar gum was added to standardize the viscosity of the incubation mixture. A value for rapidly available glucose (RAG) was obtained as the glucose released after 20 min (G20). A second measurement (G120) was obtained as glucose released after further 100 minutes incubation. A third measurement (total glucose; TG) was obtained by gelatinization of the starch in boiling water and treatment with 7M aqueous KOH under ice, followed by complete enzymatic hydrolysis with amyloglucosidase. Free glucose (FG) was also determined by treating the sample with acetate buffer and placing the tube in a water bath at 100℃ for 30 min.

A blank tube containing buffer, glass balls and guar gum was also included to correct for the glucose present in the enzyme mixture. Glucose was determined in all the samples using a glucose oxidase–peroxidase diagnostic kit from Megazyme international, Ireland.

Table 1: Nutritional classification of starch in foods (adapted from Englyst et al,1992)

STARCH TYPE	DEFINITION
Rapidly digestible starch (RDS)	The fraction of starch that is digested completely in the human small intestine, and is measured as the starch digested within 20 minutes of enzyme hydrolysis
Slowly digestible starch (SDS)	The fraction of starch that is digested completely in the human small intestine, but more slowly than RDS, and is measured as the starch digested between 20 and 120 minutes of enzyme hydrolysis
Resistant starch (RS)	The sum of starch and starch degradation products not digested in the human small intestine and is the difference between starch hydrolysed by 120min and total starch.

CALCULATIONS

RAG=G20
SAG=G120-G20
TS=TG x 0.9
RDS = (G20-FG) x 0.9
SDS = (G120-G20) X 0.9
RS = TS-SDS
SDI = RDS/TS X 100

RESULTS AND DISCUSSION

Table 2 presents the starch fractions obtained from raw and processed plantain as derived *in vitro* from the glucose fractions. Resistant starch (RS) content in uncooked samples was 36.6±1.1g/100g and 62.7±0.9g/100g for ripe and unripe samples respectively. RS reduced dramatically in the processed samples ranging between 3.5-6.5 g/100g in the ripe samples and 15.0 - 19.9 g/100g in the unripe samples. Dramatic changes observed in the levels of resistant starch in the processed samples is not unconnected with gelatinisation of starch granules which affects starch digestion, however, many other factors such as botanic origin, food form, composition, starch retrogradation and ripeness also have an impact on the levels of resistant starch (Bello-Perez, 2009, Englyst & Cummings, 1986).
Slowly digestible starch (SDS) values were between 0.6 and 9.2 g/100g and did not record the same trend as observed for the levels of resistant starch but varied between the ripe and unripe samples. SDS has been associated with the advantage of a slow increase in postprandial blood glucose levels and sustained blood glucose level over time as well as moderate impact on glycemic index, but the effect of hydrothermal treatment in the generation of slowly digestible starches are quite complex and its structural properties are still being studied (Lehmann, 2007).

TABLE 2: Glucose fractions and starch fractions for ripe and unripe plantain (g/100 dry weight basis)

sample	FG	RAG	SAG	RDS	SDS	RS	TS
Unripe raw	0.7^{d}±0.0	4.5^{g}±0.2	11.6^{a}±1.3	3.4^{f}±0.2	10.4^{a}±1.2	62.7^{a}±0.9	76.5^{bc}±0.1
Unripe boiled	0.5^{d}±0.0	58.5^{b}±1.5	7.0^{c}±1.5	52.2^{b}±1.3	6.3^{c}±1.3	16.7^{e}±2.6	75.2^{d}±0.1
Unripe boiled S	0.5^{d}±0.0	59.3^{b}±2.0	10.3^{ab}±1.3	52.9^{b}±1.8	9.2^{b}±1.1	15.0d^{e}±2.4	77.1^{b}±0.1
Unripe grilled	0.7^{d}±0.0	53.9^{c}±2.3	10.6^{b}±1.6	47.9^{c}±2.1	9.1^{ab}±1.4	19.3^{c}±2.2	76.2^{c}±0.2
Unripe fried	0.5^{d}±0.0	63.4^{a}±1.7	5.4^{d}±0.1	56.6^{a}±1.5	4.9^{d}±0.1	17.3^{cd}±1.6	78.7^{a}±0.0
Ripe raw	7.6^{a}±0.3	9.3^{t}±0.3	5.5^{d}±1.1	1.5^{t}±0.4	5.0^{d}±1.0	36.6^{b}±1.1	43.1^{e}±0.4
Ripe boiled	5.4^{c}±0.2	42.3^{d}±1.6	0.7^{f}±0.1	33.2^{d}±1.7	0.6^{f}±0.1	3.4^{g}±0.3	37.3^{f}±0.3
Ripe boiled S	5.2^{c}±0.3	40.4^{d}±2.5	3.2^{e}±0.3	31.7^{d}±2.4	2.9^{e}±0.3	3.4^{g}±0.5	38.0^{f}±1.1
Ripe grilled	5.7^{b}±0.3	35.1^{e}±2.5	3.8^{e}±0.3	26.5^{e}±1.6	3.4^{e}±0.2	6.5^{f}±0.4	36.4^{g}±0.8
Ripe fried	5.8^{b}±0.3	34.2^{e}±1.6	6.2^{cd}±0.4	25.6^{e}±1.7	5.5^{cd}±0.4	6.2^{f}±0.2	37.3^{f}±0.4

Values are means of triplicate
Boiled S- plantain boiled with skin
Values with different superscripts in the same column are significantly different while values with the same superscript in the same column are not significantly different (at 95% confidence level).

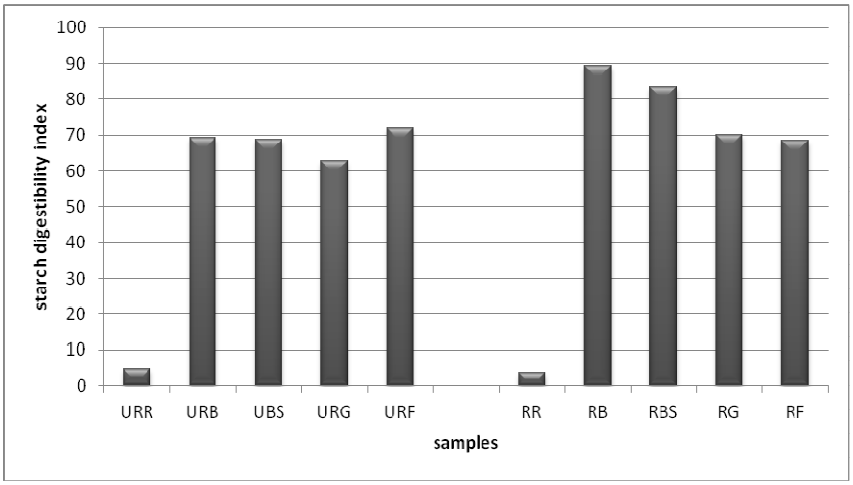

Figure 1 Effect of processing starch digestibility indices for ripe and unripe plantains
Abbreviations: UR-unripe, R-ripe, B-boiled, BS-boiled with skin, G-grilled, F-fried

Starch digestibility index (SDI), is the ratio of the rapidly digested starch (RDS) to the total starch content. Higher starch digestibility index (SDI) in processed samples (figure 1), which implies higher digestibility, may be an indication of the extent of starch gelatinization. Amongst the processed samples, the highest SDI value was recorded in the ripe boiled sample (90) while the lowest value was in the grilled unripe sample (60). This can be attributed to the dry-heat processing conditions of grilling and lower moisture involved during grilling as compared with boiling. Starch gelatinisation is dependent on several factors amongst which the starch-moisture content plays a significant role.

Rapidly available glucose (RAG) represents the quantity of glucose that is available quickly for absorption after a meal. The RAG is the sum of rapidly digestible starch (RDS) and free glucose present in the food. Hence, it is useful in predicting the glycemic response to carbohydrate foods as proposed by Englyst *et al* (1999).

CONCLUSION

Ripe plantain contained significantly lower amounts of all nutritional starches which further decreased with processing when compared with unripe plantains. This information will be useful in developing food processing conditions that positively affect starch resistance to digestion in order to optimize its nutritional quality and to improve the physiological benefits.To maximize resistant starch (RS) and slowly digestible starch (SDS) contents in plantains, it would be important to identify the stage of ripening and the processing techniques needed to develop desired products which are fit for consumption, and for delivering optimum benefits required.

ACKNOWLEDGEMENTS

This work forms part of a PhD research and Ebun-Oluwa Oladele is grateful to the Commonwealth Scholarship Commission for sponsorship.

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INFLUENCE OF PRE-TREATMENT ON YIELD AND QUALITY OF MANDARIN JUICES

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ABSTRACT: Croatia in the Neretva valley produces remarkable quantities of mandarins but, as always, one part of yield, according to fruit characteristics, are not suitable for consumption as fresh. At the same time those fruit are suitable for processing industry e.g. for juice production. In Croatia, mandarin juice is not industrially produced, and generally is rare on market. The current study is contribution to mandarin juice processing with the aim to investigate influence of different pre-treatment on yield, quality, biological and sensorial attributes of obtained juices.
Mandarin from Neretva valley was used to produce cloudy juices on small scale equipment. Before pressing, fruits were peeled and treated (i) with maceration enzymes at various temperature and duration (EPT) (ii) by ultrasound (USPT) with different amplitude and duration. Control juices were produced without enzymes or USPT. In juices biologically active compounds (BAS) total carotenoids (TC), total phenolics (TP), and vitamin C were determined. All juices were sensory evaluated (SE) by quantitative descriptive method. The results were statistically analyzed. Considering the investigated parameters there are no remarkable differences among juices produced by enzyme treatments independent of enzyme concentration, temperature (20 or 50 ^{o}C) and duration of 30 or 60 min. regarding to BAS and SE. Also, different US treatment conditions did not show any remarkable influence on investigated parameters. But in comparison of these two types of treatment better yield and SE were obtained by US and higher content of TC was obtained by enzyme treatment. Treatment by US could be recommended to produce mandarin juice due to high yield and SE but also further investigations are needed to optimize US conditions due to better preservation of BAS.
Key words: *mandarin juice, ultrasound, enzyme maceration, biologically active compounds*

INTRODUCTION

Croatia in the Neretva valley produces remarkable quantities of mandarins but, as usual, one part of yield, are not suitable for consumption as fresh. At the same time those fruit are suitable for processing industry e.g. for juice production. In Croatia, mandarin juice is not industrially manufactured, and generally is rare on the market. Looking on juices from citrus fruit, on Croatian market, as well as on world market, orange juice is predominant. In comparison to orange juice mandarin juice has higher intensity of orange colour and may be added to orange juice to improve its colour (Perez-Lopez et al., 2006). Citrus juice production usually is based on extraction from unpeeled fruit and peel constituents which may pass into the juice later in technological process it should be removed (Bates et al., 2012). Since, mandarin is easy-to-peel fruit, mandarin juice could be produced with previous peeling (Tietel et al., 2010) what is convenient for SMEs (Bates et al., 2012) because peel constituents are not present in fresh juice after extraction which technologically simplifies finalization of juice. Yield of juices obtained by pressing is in dependence on pectin structure and content therefore pectolitic enzymes usually are used. To obtain higher yield certain enzyme treatment before pressing is desirable for many fruit (Chang et

al., 1994, Levaj et al, 2010, Levaj et al., 2011). Recently in juice production, ultrasound technology has been investigated mostly as an substitution to common thermal treatment e.g. pasteurization in order to inactivate enzymes and microorganisms at mild temperature conditions (Tiwari et al., 2008; Dubrović et al., 2011; Rawson et al., 2011; Chandrapala et al., 2012; Fonteles et al., 2012) but in laboratory ultrasound is well-established as method for cell disintegration to extract intracellular components (Skauen, 1976, Valero et al., 2007). High-intensity cavitation punctures the cell walls and releases the cell content. Mandarin as other citrus fruit contain high valuable nutrients such as vitamin C, folate, dietary fiber, minerals (potassium) and biological active comounds (BACs), the terpenes and phenolic compounds, which synergistic action with vitamin C contribute to citrus antioxidant capacity (Codoner-Franch and Valls-Belles, 2010; Unno et al., 2011). Mandarin is rich in hesperidin, flavanon specific for citrus fruit, and with carotenoid, β-chryptoxanthin, which possess anti-inflammatory and anti-carcinogenic activity (Codoner-Franch and Valls-Belles, 2010; Kohno et al., 2001). Flavonoids also have a role in cardiovascular protection, (Codoner-Franch and Valls-Belles, 2010). Consquently, better releasing of hesperidin and β- chriptoxanthin, generally speaking, phenolics and carotenoids, in the processing of juice are desirable in order to better quality as well as health reasons. The current study is contribution to mandarin juice processing with the aim to investigate influence of ultrasound in comparison to thermal and enzyme treatment on yield, quality, biological and sensorial attributes of obtained juices.

MATERIAL AND METHODS

Mandarin fruit (*Citrus unshiu* Markovich), were harvested, twice with an interval of two weeks, in the Neretva Valley in 2010 and transported at 4 ℃ to Faculty of Food Technology and Biotechnology in Zagreb, hand peeled and frozen at -18 ℃ till juice manufacturing.

Juice manufacturing

After defrosting fruit was chopped by knife or homogenized in blender (Mixy Zepter, International) and was used to produce cloudy juices on small scale equipment according to scheme (Figure 1).

Ultrasonic pre-treatment (USPT)

Homogenized fruit (round 400 mL) was placed in a glass beaker (1000 mL), which served as the treatment chamber. An ultrasonic processor (S-4000, Misonix Sonicators, Newtown, CT, USA), set at 600 W, 20 kHz and 12–260 μm with a 19-mm diameter probe, was introduced into the vessel. Ultrasonication was carried out with 60 and 120 μm amplitude. Samples were treated ultrasonically for 5 and 10 min at 25 ℃ (table 1). Each experiment was conducted at least in duplicate.

Thermal (TPT) and enzyme pre- treatment (EPT)

Chopped fruit for was treated at 20 or 50 ^{o}C for 30 minutes (TPT) and for EPT with 50 or 150 ppm maceration enzymes (Endozym Pectofruit PR, AEB group, Italy) at 20 or 50 ^{o}C for 30 or 60 minutes (table 2). These concentrations and temperatures were selected according to the manufacturer's instructions.

Pressing was done on hydraulic pack press (Euclid Ltd., Croatia). Juices were kept in refrigeration at 8^{o}C until analysis. Juices were produced separately with the mandarin from the first harvest and with the mandarin from the second harvest. Each juice was separately analyzed.

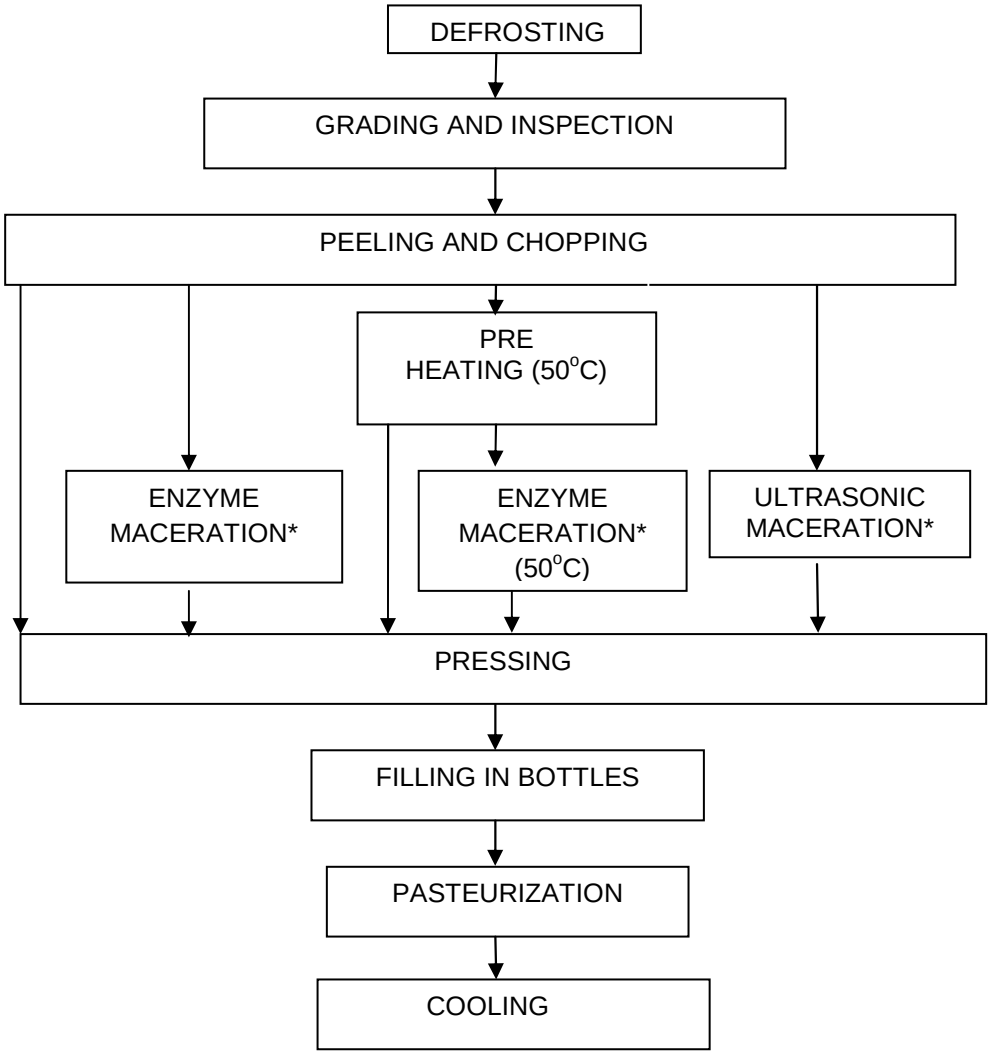
Table 1. Conditions of USPT and the code of produced juices

Code of sample	Amplitude/μm	Duration (min)
US/60/5	60	5
US/60/10	60	10
US/120/5	120	5
US/120/10	120	10

Table 2. Conditions of TPT and EPT and the code of produced juices

Code of sample	T (℃)	Enzyme (ppm)	Duration (min)
20/0/30	20	-	30
20/50/30	20	50	30
20/150/30	20	150	30
20/50/60	20	50	60
20/150/60	20	150	60
50/0/30	50	-	30
50/50/30	50	50	30
50/150/30	50	150	30

After pressing juice was filled in glass bottles of 200 mL and closed. Pasteurization of juices was done after filling at 85 ^{o}C/15 min. Cooling was done by immersing in cold water.
Yields were calculated on fruit pulp, after peeling.



*-see table 1 and 2

Figure 1. Scheme of juice manufacturing

Biologically active compounds determination and sensory evaluation

Total phenolics (TP) were extracted according to Coseteng and Lee (1987) and determined by spectrophotometer using Folin-Ciocalteu reagent (Ough and Amerine, 1988) based on gallic acid calibration so results are presented as equivalent of gallic acid (GAE).

Carotenoids were extracted by petroleum ether (V_u=total volume of extract (mL)), absorbance (λ=450 nm) ($A_{\lambda max}$) was measured by spectrophotometer and concentration was calculated by using extinction coefficient $E^{\%}_{1\ cm}$ (extinction coefficient in petroleum ether for β-carotene=2500) and formula (Vuelleumier, 1967):

$$\text{Total carotenoids (as β-carotene) (mg/100 g)} = \frac{A_{\lambda max} \cdot 1000 \cdot V_u}{m(g) \cdot E^{\%}_{1\ cm}}$$

Vitamin C were determined by titration with 2,6-diclorphenolindophenol (AOAC, 2002).

All analyses were done in duplicates.

Obtained juices were conducted to sensory evaluation by Quantitative descriptive analysis which was very comprehensive. The panellists were requested to list the terms appropriate to describe the colour, odour, taste, consistency and overall

sensory impression whereas a total of 10 descriptive terms for all major sensory attribute categories were generated. The panellists scored the samples using a suitable line intensity scale, with scores awarded on a scale of 0 – 10 in which 10 indicated the highest intensity of evaluated sensory attribute. Sensory analysis was carried out by a trained panel consisting of fifteen members per session. The procedure was performed according to ISO 6564, ISO 8587 and ISO 11036 (in a sensory laboratory equipped according to ISO 8589) and in consistence with method from literature (Bursać et al., 2007; Bursać Kovačević et al., 2008).

Statistical data analysis

Statistical analysis was performed via analysis of variance (ANOVA) by Kruskal-Wallis test using SPSS (ver. 17) in order to investigate influence of pre-treatment (temperature, enzyme – temperature, and ultrasonic) on each determined parameter. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

All treatments were grouped as follow: (i) thermal pre-treatment (TPT) in order to investigate influence of temperature, (ii) enzyme pre-treatment (EPT) in order to investigate influence of enzyme concentration, temperature and duration of maceration, and (iii) ultrasonic pre-treatment (USPT) in order to investigate amplitude and duration of ultrasonic treatment.

Within each treatment there was not significant influence of temperature or enzyme concentration, temperature and duration of maceration or amplitude and duration of ultrasonic treatment on juice yield. However, USPT ranked highest yield followed by EPT and TPR (χ^2=11.60, p<0.01). Average values of juice yield are shown in table 3. Lieu and Le (2010) investigated US in grape mash treatment and also concluded that sonication treatment increased extraction yield.

Table 3. Average values of each treatment for yield, TP, TC and vitamin C

Treatment	Yield (%)	TP (mg/100 g)	TC (mg/100g)	Vitamin C (mg/100 mL)
TPT	65.27±6.09	76.45 ±26.98	0.75±0.26	17.18±4.16
EPT	75.55±6.75	73.36±37.55	0.75±0.11	17.81±2.11
USPT	83.16±5.97	71.07±11.38	0.44± 0.11	19.66±2.83

Influence of all treatments on BACs (TC, TP and vitamin C) was investigated. Content of TP were a little lower than previously determined in fresh mandarin (114.09 mg/100g) (Levaj et al., 2008) which may be due to a difference in the mandarin cultivars and the processing. According to average values for each treatment (table 3) the highest TP content was obtained by TPT and retention in USPT was 93 %. With prolonged time and higher amplitude loss of BACs was higher although the differences were not significant (table 4). Rawson et al. (2011) had similar observation for watermelon. TP content was not much affected at lower processing times of 0 to 6 min in comparison to higher processing times of 10 min where significant degradation of TP content was observed. Fonteles et al. (2012) also conclude that ultrasound caused reduction of TP content. In our study significant influence of pre-treatment was observed only for TC content (χ^2= 14.14, p=0.01). The average value for USPT was 40 % lower when compared to the highest average value of TC content determined for EPT (table 3). Although between all treatments there was no significant influence on vitamin C content, its average value of USPTs slight increased in comparison to other two treatments (table 4). Rawson et al. (2011) reported about degradation of vitamin C as processing time was increased from 0 to 10 min for an amplitude level of 24.1 μm at 25 ℃. In our study at lower amplitude was achieved higher vitamin C content which decreased with the higher amplitude but the differences were not significant (table 4).

Table 4. Average values of each USPT for TP, TC and vitamin C

USPT	TP (mg/100 g)	TC (mg/100g)	Vitamin C (mg/100 mL)
US/60/5	76.66±4.72	0.42±0.01	22.26±0.49
US/60/10	72.78±18.07	0.45±0.19	21.92±0.98
US/120/5	75.37±1.84	0.44±0.16	16.70±2.45
US/120/10	59.45±13.36	0.47±0.13	17.74±0.0

Additionally, within each treatment there was no significant influence of temperature or enzyme concentration, temperature and duration of maceration or amplitude and duration of ultrasonic treatment on TC, TP or vitamin C content.

(a) (b)

Figure 2. Results of sensory evaluation: (a) desirable attributes; (b) undesirable attributes

All sensory attributes are divided in two main evaluation blocs: first one include desirable attributes (see Fig. 1a) and the second one undesirable attributes (see Fig. 2b). Total sum of scores for desirable attributes and total sum of scores for undesirable attributes were calculated and mathematically adjusted that both blocks had the same share in total sensory score regardless of number of grades in blocs and than were subtracted. For desirable attributes (χ^2= 37.84, $p<0.01$) as for TSG (χ^2=54.53, $p<0.01$) the best ranking treatment was USPT, while TPT has the largest rank for undesirable attributes (χ^2=53.78, $p<0.01$).

CONCLUSIONS

Ultrasound is promising method in juice processing as a pre-treatment method to ensure alternative of thermal or enzymatic method according to results of sensory evaluation and yield. Additionally, better yield in this study was obtained in much shorter time when compared to conventional methods. Further investigations are needed to improve content of BAC in juices.

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EFFECT OF THE PARAMETERS OF OSMOTIC DRYING ON SOME PHYSICAL PROPERTIES OF QUINCE

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ABSTRACT: Osmotic drying of quince is used as the first step in combined drying. The removal of moisture during osmotic drying ensures less time for convective drying. Osmotic treatment reduces water activity, provides microbiological stability and extended shelf life of products. In addition, osmotic pre-treatment has a positive effect on the mechanical properties of the final product. The impact of osmotic drying on the change of quince color and volume was examined. Quince cubes of dimensions 15x15x15 mm were used in the experiment. Osmotic drying was carried out with the following combinations of temperature and concentration of sucrose solution in water: 40°C,50°Bx; 60°C,50°Bx; 40°C,65°Bx and 60°C,65°Bx. Drying time was 180 minutes. The samples were tested every 20 minutes. The samples, treated by osmotic drying, were air dried convectively at the temperature 40°C for 20 hours. Color measurement was performed by Konica Minolta colorimeter CR-400. CIE L*a*b * values were used for color parameters. During osmotic drying significant changes in parameter b* were measured, while the parameters L* and a* did not change significantly. At high values of temperature and concentration of osmotic solution less color change occurs after convective drying. The biggest changes in volume were measured again at the highest values of temperature and concentration of osmotic solution.
Keywords*: quince, osmotic drying, color, volume, dimensions*

INTRODUCTION

Quince is a fruit species that is highly appreciated for its aroma. It is typically used in the production of brandy and compotes, while it is rarely used in the fresh state. This is because of the hardness and firmness of the fruit (Babić et al, 2008). Dried quince is used as an ingredient in muesli and tea blends. At the Faculty of Agriculture in Novi Sad a new quince product has been developed to be used for direct consumption as a delicacy. This product is a result of combined methods of drying. Combined drying consists of osmotic and convective drying. The advantages of combined drying, apart from the improvement of mechanical properties, are reflected in preserving the quality of dried fruit (color, flavor, aroma and taste), prolonged periods of storage with higher final humidity and shorter drying to final moisture content (Babić, Ljiljana and Babić, M., 2003). During the drying process the physical properties of materials change. The aim of this study was to determine the effect of different parameters of osmotic drying on the change of the color, size and volume of quince. Food drying is one of the most common ways of protecting and preserving the stability of food (Mayor and Sereno, 2004). However, during drying, the material is exposed to high temperatures which can have adverse effects on the quality (Barreiro et al, 1997; Ibarz and Lozano, 1997; Avila and Silva, 1999; Ibarz et al, 1999). However, some authors reported that osmotic drying reduces these changes. The first indicator of product quality is its color and shape. Change of the color of fruit may be due to chemical, biochemical and physico-chemical mechanisms: degradation of cells, changes of natural pigments (chlorophylls, carotenoids and anthocyanins) and development of enzymatic browning. Drying and water loss cause changes of cell microstructure and volume (Koc et al., 2008). The size of these

changes depends on the moisture transport mechanism. That is the method and drying conditions affect the intensity of volume changes. Convective drying is the most commonly applied method of drying. During this drying method the volumes of material significantly change and the rate of rehydration is reduced. Osmotic pre-treatment has the positive influence on preservation of shapes and sizes.

MATERIALS AND METHODS

A variety of quince Leskovačka was used in the experiment. On the basis of previous research results this variety is very suitable from the aspect of size and shape. The final products, dried quince quarters, have a satisfactory shape and dimensions. These properties are important because the research has been focused on developing products for direct consumption (Radojčin et al., 2010). Color measurement was performed on quince cubes of the dimensions 15x15x15 mm. The cubes are formed in quarters and treated with sulfur dioxide. The measurement was carried out on three samples, each time on the same area. The values, shown in Figures 1, 2 and 3, represent the average of the three measurements. Magdić and Dobričević (2007) reported that color measurement can be nonobjective because it is not possible to capture the same area. The shape and dimensions of the samples enabled the recording of quince colors always on the same place. The measurement was performed on the beginning of the process and after every 20 minutes during osmotic drying. Before each measurement the excess of osmotic solution was removed from the samples. Osmotic drying was carried out in a solution of water and sucrose. Drying time was 180 minutes. Osmotic drying was carried out at the following combinations of temperature and sucrose solution concentration: 50^{o}Bx and 40^{o}C, 60^{o}C and 50^{o}Bx, 40^{o}C and 60^{o}C and 65^{o}Bx and 65^{o}Bx. After osmotic drying, convective drying was carried out in an experimental dryer with trays. Convective drying time was 20 hours at the temperature of 40^{o}C, for all samples.
Color measurement was performed by Konica Minolta colorimeter CR-400. The value of the colors is shown in the CIE L*a*b* color system. This way of representing the color is suitable because the measured value of the color is shown in three-coordinate system. In this system L* indicates lightness (brightness, light), a negative value a* is green, and positive a* is red. Negative b* is yellow, a positive b* is blue (Alvarez-Fernandez et al, 2003).
For presentation of color change the value ΔE*ab is also used. This value represents the total color change. This is the difference between the color at the beginning of measurements and at appropriate moment during the experiment.

$$\Delta E^{*}ab = \sqrt{\left(L_o^{*} - L^{*}\right)^2 + \left(a_o^{*} - a^{*}\right)^2 + \left(b_o^{*} - b^{*}\right)^2}$$

where: L_o*, a_o* b_o* are the initial values measured on fresh fruit samples, and L*, a*, b* are values measured after drying process (osmotic, convective or combined).
After convective drying, the color of samples was measured, too. The color measurement was conducted on the samples which were not osmotically treated. The color of these samples was measured after sulphuring and after convective drying.
Based on Archimedes' law the sample volume was determined. In order to measure the volume it is necessary to have a scale, glass gauge and a thin metal wire (Babić, M., and Ljiljana Babić, 2007). To calculate the volume it is necessary to measure the mass glass gauge with distilled water before and after the sample is submerged in water. The volume of the sample is calculated using the following equation:

$$V = \frac{M_{II} - M_I}{\rho}$$

where: V - quince cube volume [m^3], M_I - mass of glass with liquid [kg], M_{II} - mass after immersing the sample in glass [kg], ρ - the density of distilled water [kg/m^3]
Shrinkage is the relation between the changes in volume of the sample at a certain moment during drying and the volume at the beginning of drying.

$$SV = \frac{V_0 - V_i}{V_0} \cdot 100(\%)$$

where: - SV [%] – shrinkage, relative change in volume, V_o - initial volume [m^3], V_i - volume at a certain moment during drying [m^3].

RESULTS AND DISCUSSION

During osmotic drying the color parameter b*, which means yellow color, changed during all 180 minutes. There is a constant increase in the value of this parameter. Somewhat larger change of this parameter was measured at the concentration of 65^0Bx. The values of parameters L^* and a^* have not significantly changed. Smaller decline and growth of these values is noticeable. The value of parameter L^* is in the range from 70 to 80, while the value of the parameter a* ranged from -6 to -4. Osmotic drying provides the color of the final product very similar to fresh fruits, what was also confirmed by Rodrigues et al. (2003).

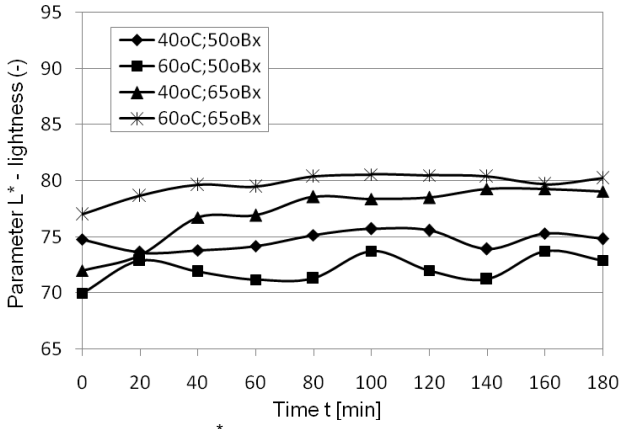

Figure 1. Change of color parameter L^* during osmotic drying for different osmotic parameters

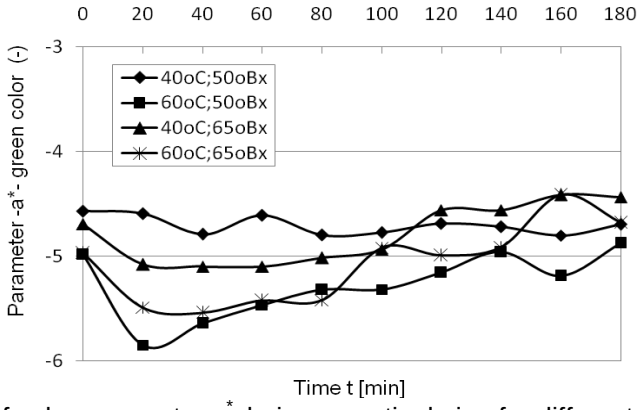

Figure 2. Change of color parameter a^* during osmotic drying for different osmotic parameters

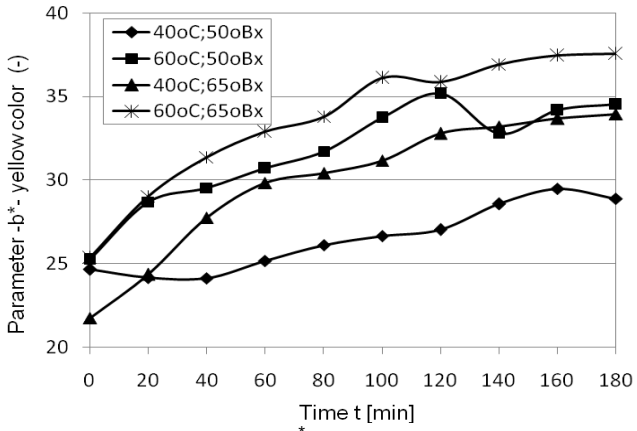

Figure 3. Change of color parameter b^* during osmotic drying for different osmotic parameters

The mean values of color parameters L^*, a^* and b^* in the quince samples which were not osmotically dried are shown in Figure 4. L^* and a^* parameters did not change significantly. These measured values changed only for 2 or 4 units. The more significant change of parameter b^* was measured. The difference between parameter b^*, for fresh and convectively dried samples, was 10. It can be said that this difference is significant.

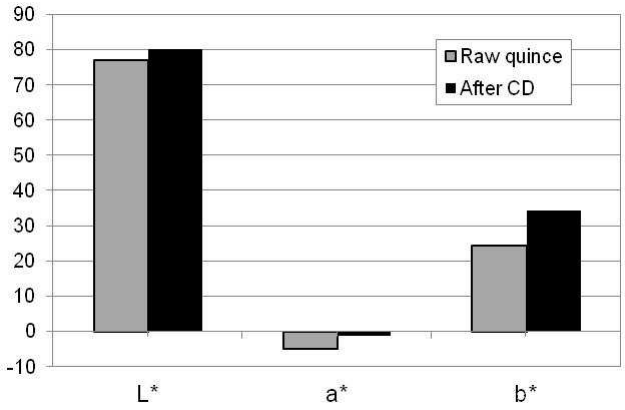

Figure 4. Values of color parameters (L*,a*,b*) of raw quince and after convective drying (CD)

The total color change ΔE*ab is a parameter that is often used to evaluate color changes in food products. In all the experiments the total color change was recorded (Figure 5). Somewhat larger changes were measured at the solution concentration of 65°Bx. Some authors suggest that less moisture in the product is favorable for the progress of the tanning process. This could explain such a result. However, an unexpected result was the greatest color change at the temperature of 40°C and the solution concentration of 65°Bx. After convective drying, smallest differences of total color were measured at the temperature of 60°C and the solution concentration of 65°Bx. The formation of the sugar layer on the surface of the sample prevents any chemical reaction with the surrounding air which results in browning of fruit tissues (Rahman and Mujumdar, 2007). Less change in total color was measured in quince samples which were not osmotically dried. One of the goals during food processing is to preserve the color. However, the total color change in the experiments with osmotic drying was caused by changing of the parameter b^* (Table 1). There was no significant change in the parameter L^*, the lightness was preserved. The result is a product of intense and purer yellow color.

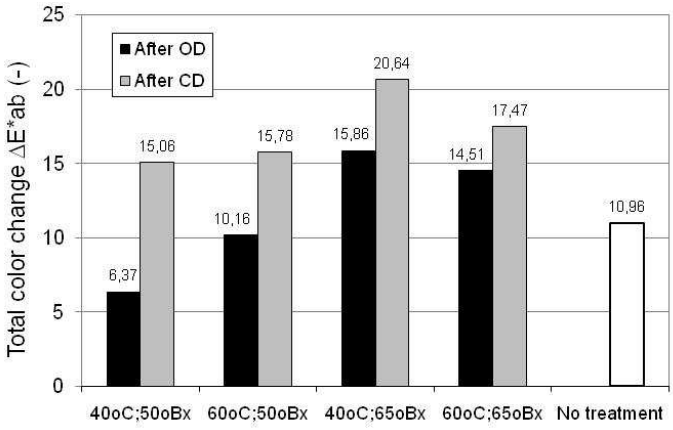

Figure 5. Total color change after osmotic and combined drying (■ ■ osmotic treated quince, □ convectively dried quince-without osmotic treatment)

Table 1. Color parameters of raw quince, after OD, after CD (combined drying) for different osmotic parameters

Material	Color parameters	40°C; 50°Bx	60°C; 50°Bx	40°C; 65°Bx	60°C; 65°Bx
Raw		74.80	70.15	72.05	77.08
After OD	L^*	74.81	72.92	79.05	80.22
After CD		77.72	69.94	77.79	78.03
Raw		-4.57	-4.99	-4.69	-4.97
After OD	a^*	-4.69	-4.87	-4.43	-4.68
After CD		-2.24	-1.08	-1.61	-2.14
Raw		24.66	25.24	21.74	25.35
After OD	b^*	28.89	34.52	33.94	37.56
After CD		39.08	42.25	40.19	41.64

OD-osmotic drying, CD-combined drying

Volume change during drying is an important indicator of product quality. It depends on the type of materials, cell properties and structures and drying conditions. Experimental data published in some studies show that the volumetric shrinkage is dependent on the moisture content in the material (Lozano, Rotstein, & Urbicain, 1983; Suzuki, Kubota, Hasegawa, & Hosaka, 1976). Lozano et al. (1980) describe the shrinkage of apples in the function of moisture content as a linear model. However, non-linear models adequately describe the volumetric shrinkage of fruits and vegetables. The values of shrinkage during osmotic drying are given in the diagram (Figure 6). The biggest changes were measured for the solution concentration of 65°Bx. The change intensity is the greatest at the beginning and it decreases during drying. The change of the moisture content is similarly changed during drying, which indicates the relationship between changes in volume and moisture. This relationship between volumetric shrinkage and moisture content is not linear. This dependence is best described by the nonlinear model. These results are reported Babić et al. (2008). Volumetric shrinkage ranges from 33% to 53% for different parameters of osmotic drying.

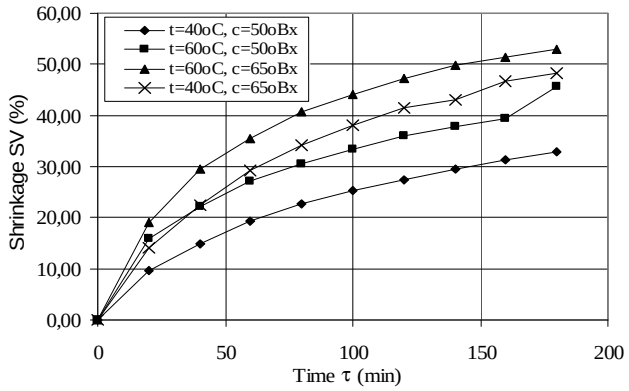

Figure 6. Volumetric shrinkage during osmotic drying

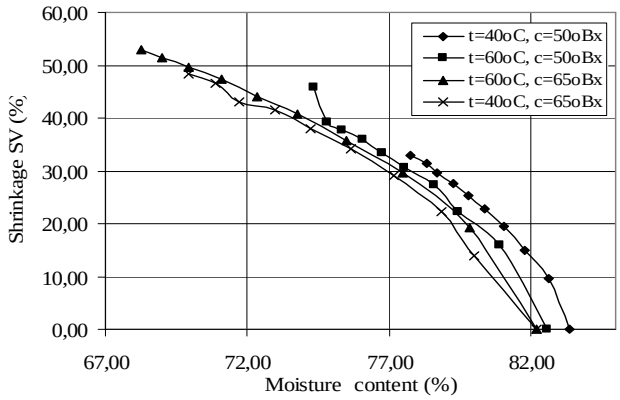

Figure 7. Shrinkage as a function of moisture content during osmotic drying

CONCLUSION

The physical properties of food products are the first indicator of quality. Preserving these properties is one of the goals to be achieved during the drying process. The change of the total color of quince occurs during osmotic drying. Less change of color after convective drying occurs at high values of temperature and osmotic solution concentration. A layer of sugar on the surface of the sample has a protective role in preserving the color of products. The biggest change in volume and dimensions were measured again at the highest values of temperature and concentration of osmotic solution. Changing the volume and dimensions, depending on moisture content and drying time of material, is best described by the nonlinear model. Further research should be directed towards the search for dependencies between the studied parameters and other parameters that affect their behavior.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31058, 2011-2014. financed by the Ministry of Science and Technology, the Republic of Serbia.

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OSMOTIC DRYING KINETICS OF NECTARINE HALVES (*Pyrus Persica L.*)

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ABSTRACT: The experimental research of the osmotic drying kinetics of nectarine tissue in a sucrose solution has been conducted. As the research material, the mesocarp tissue of the nectarine variety *Fantasia* has been used in the shape of fruit halves. Based on the data obtained by a dispersion analysis of this two-factor experiment (with the statistical probability of 99%), it can be concluded that there are impacts of the selected factor levels on the drying speed of the nectarine tissue and the dry matter increase. The most significant moisture decrease, dry matter increase, and ***volume shrinkage*** has been noted in the combination t_r=60^{o}C and C_r = 65^{o}Bx, (2.27 kg_w/kg_{sm}; 3.78%, 0.3449, successively), whereas the least significant changes have been noted in the combination t_r = 40^{o}C and C_r = 50^{o}Bx (1.45 kg_w/kg_{sm}, 2.45%, 0.174, successively).Using a regression analysis, a mathematical modelling of moisture change (compared to the dry base) and dry matter increase of the nectarine tissue was conducted during the osmotic drying. Three empirical models, enhanced with factors of non-dimensional osmotic drying, have been selected for every modelled value. Based on the statistical indicators such as the correlation coefficient (*R*), the chi-square test (χ^2), the ***mean bias error*** (MBE), and the mean squared error (MSE), models have been assessed and the most precise models have been selected. Ultimately obtained mathematical models have high correlation coefficients (R = 0.9886 and R = 0.9936).
Keywords: *osmotic drying, drying kinetics, nectarines.*

INTRODUCTION

The research subject of this paper was the osmotic drying (mesocarp) of nectarine tissue in the form of fruit halves in a sucrose solution. In scientific literature the pieces of information on the influence of parameters of osmotic drying on changes in physical characteristics of fruit tissues of different fruit types are numerous. When it comes to the fruit type which is the subject of this research, the results are few and they refer to the tissue dried in the form of regular geometrical shapes (cube, prism, cylinder, sphere) (*Fernandez el al., 2004, Kingsly, 2007*). In production of dried fruit, as a final product, the above mentioned geometrical shapes are rarely used, except for certain types of food supplements. The results of this kind of research, apart from the scientific purpose, are rarely applicable in the design process of drying of the fruit tissue where the completely natural fruit shape is used, or a part of it (one half of the whole fruit, one quarter of the whole fruit).

The aim of the research as a part of this paper is to determine the changes of the basic physical characteristics of nectarine halves during the osmotic drying in a sucrose solution depending on process parameters. Based on the measured values, mathematical models of the changes of fruit tissue moisture in relation to the dry base and dry matter increase will be established. Mathematical models will be in function from analyzed parameters of the working fluid from osmotic drying. The established mathematical models should be as correct as possible, but at the same time simple enough for the practical usage.

MATERIAL AND METHOD

In the experimental part of the research fresh fruit of the nectarine variety Fantasia has been used. Nectarines were obtained from orchards in Srem, near Čerević, Serbia. Until the beginning of the processing, fruit was stored in a locker on the temperature of 4^{o}C and 75% relative air humidity. The stage of fruit ripeness was the beginning of technological maturity with the average values of pH = 2.99±0.14 and fruit strength of 5.55± 0.42 kg/cm^2. The measured values of the basic physical characteristics of the fruit were: moisture in relation to the dry base 4.51± 0.13 $kg_w/kg_{sm,}$ fruit mass 113.51± 15.66 g, length 60.60±2.78 mm, width 58.24±3,45 mm, and thickness 56.50±2.98 mm. All average values were measured on a random sample of 20 fresh fruit.

The preparation of fresh fruit for the drying implied washing, cutting in halves and the removal of the seeds. With this type of preparation, the usable mass of tissue was 103.75±15.78g which makes 91.25±1.84% of the whole fruit mass. Prepared halves had the following mean value of physical characteristics: tissue moisture in relation to the dry base 4.58±0.274 $kg_w/kg_{sm;}$ length of one half 56.20±3.178 mm; width of a half 54.34±2.427 mm; thickness of a half 25.37±1.686 mm; volume of a half 41.73±4.30 cm^3. After cutting in halves the prevention of tissue darkening was conducted. With the dry process of sulphuring, 2g of technical sumphur in powder per 1 kg of prepared halves was burnt. The exposure time of the halves to the sulphur dioxide in the sulphuring chamber was 8 h. Upon completing of the preparation, the samples were placed in the experimental semi-industrial osmotic dryer, with the capacity of 0.039 m^3 (Babić, M., et al., 2004, Pavkov et al., 2007). When planning the experiment one of the main criteria was to achieve the simulation of real processes of osmotic drying in the experimental dryer. For this reason, the experiment was conducted in a semi-industrial osmotic dryer.

Previous studies on the process of osmotic drying of fruit tissues and influential factors emphasize the following statement: temperature and osmotic solution concentration are the two most influential factors on the rate of mass transfer during osmotic drying (Park et al., 2002, Babić, Lj., 2007, Khoy and Hesari, 2007, Pavkov, 2007; Pavkov et al 2009). Based on these findings, the experiment was performed with two factors. Osmotic solution temperature was varied at two levels, 40℃ and 60° C and two levels of concentration of osmotic so lution, 50^{o}Bx i 65^{o}Bx. The duration of the process of osmotic drying was 180 minutes. The mass ratio of osmotic solution and nectarine halves of the sample was 10:1. Velocity of solution in front of the layer of the material 0.00913 m/s. Porous of a layer of the nectarine halves at the beginning of osmotic dehydration was 33.79 ± 1.47%. To obtain osmotic sucrose solution was used in the form of confectionery sugar crystal as solute and distilled water as a solvent. For each combination of selected levels of influencing factors of osmotic drying three repetitions were carried out.

During the experiment of osmotic drying every twenty minutes, from the start of the process until the expiry of the stipulated time, three halves were taken from the osmotic dryer. It is assumed that these samples represent the mean changes in physical properties of fruit tissue during osmotic drying in the observed time period. Their moisture content, increase in dry matter and the volume were measured.

Samples and increased moisture content of dry matter was measured by thermogravimetric method, using a laboratory dryer, Sterimatic ST-11, “Instrumentaria”, Zagreb and analytical scales KERN 440-33N (0 - 200 g, 0.01g, ±0.02) in three repetitions (Službeni list, 1983). Calculation of dry matter increase was based on the equation (*Bchir et al., 2009; Togrul and Ispir, 2007; Park et al. 2002*):

$$SG = \frac{m_{dm(i)} - m_{dm(0)}}{m_0} \quad (1)$$

where is: *SG* – solid gain (kg_{dm}/kg); m_0 – mass of fresh sample (kg); $m_{dm(0)}$, $m_{dm(i)}$ – mass of sample dry matter: at the beginning of the process, at the *i* - moment of drying process (kg_{sm});
Volume of the sample (half or quarter) was measured using the analytical scale KERN 440-47N (0 -2000 g; 0.1 g, ±0.2 g), glass cylinders and thin metal wires (*Babić, M. et al., 2007, Mohssenin, 1980; Rahman, 2009*). Measured values of the volume of individual pieces of fruit tissue before osmotic drying and in the moment of osmotic drying were used to calculate the shrinkage in volume according to the equation (*Babić, M., et al., 2008*):

$$SV = \frac{V_{(0)} - V_{(i)}}{V_{(0)}} \quad (2)$$

whre is: *SV* – volumetric shrinkage (-); $V_{(0)}$ $V_{(i)}$ – volume of halves: before drying, at the i-moment of drying process (cm^3).

Measuring the temperature of the solution during the experiment was carried out every 20 minutes using a mercury thermometer (0 - 110^{o}C, 0.1^{o}C, ±0.05^{o}C), and solution concentration using a digital refractometer ATAGO– Japan, model PAL – α (0-85^{o}Bx, 0.1 ^{o}Bx, ±0.1^{o}Bx). Velocity of osmotic solution was measured using an ultrasonic flowmeter, Krohne UMF 600P, sensor A, USA (0.03 – 13.47 m/s, 0.01 m/s, ±0.02 m/s). Dimensions were measured using the fruit floating measure TMA INOX, D-6-1, Nemačka (0 - 200 mm, 0.1 mm, ±0.05 mm).
The influence of the chosen levels of temperature and osmotic solution concentration on the rate of nectarine halves moisture change and dry matter increase was checked by the dispersive analysis of the double-factor experiment with a statistical probability of 99%. The influence of the chosen factor levels was analyzed on the basis of the calculated mean speed of drying of nectarine halves and the mean speed at which dry matter increases for the drying period of 180 minutes. Dispersion analysis was performed in the program package Statistica 10 (*StatSoft, Inc., 2010*).
Based on the results of the experiment, what was carried out was modeling of changes in moisture with regard to the dry base during osmotic drying, and modeling of increase in dry matter in comparison to the wet base during the drying time. For both sizes that were modeled three mathematical models were adopted as a starting point based on the literature review, theoretical, half-theoretical and empirical models of fruit drying kinetics. (Henderson and, Perry, 1976; Yagcioglu et al., 1999, Wang and Singh, 1978; *Panagiotou, et al, 1998*). In accordance with the aim of this research, the initial models were expanded in the function of influencing factors of the experiment in dimensionless form, namely as the ratio of the selected factor levels and its reference value (*Babić, Lj., et al., 2004*; *Panagiotou, et al., 1998;Pavkov, 2007*). The proposed equations for the change of moisture of nectarine halves in relation to the dry base are: 3, 4, 5; and the equation for the increase in dry matter are: 6, 7 i 8. The values of osmotic solution concentration and temperature of osmotic solution as a reference value were adopted, and those amounts are: $t_{r(ref)}$ = 60^o*C* i $C_{r(ref)}$ = 65^o*Bx.*

- $$\omega = a \cdot \exp(-k \cdot \tau) \cdot \left(\frac{t_r}{t_{r(ref)}}\right)^{n_1} \cdot \left(\frac{C_r}{C_{r(ref)}}\right)^{n_2} \quad (3)$$

- $$\omega = a \cdot \exp(-k \cdot \tau) \cdot \left(\frac{t_r}{t_{r(ref)}} \right)^{n_1} \cdot \left(\frac{C_r}{C_{r(ref)}} \right)^{n_2} + c \quad (4)$$

- $$\omega = a + \left(b \cdot \tau + c \cdot \tau^2 \right) \cdot \left(\frac{t_r}{t_{r(ref)}} \right)^{n_1} \cdot \left(\frac{C_r}{C_{r(ref)}} \right)^{n_2} \quad (5)$$

- $$SG = a \cdot \tau^{n_1} \cdot \left(\frac{t_r}{t_{r(ref)}} \right)^{n_2} \cdot \left(\frac{C_r}{C_{(r)ref}} \right)^{n_3} \quad (6)$$

- $$SG = \exp(k \cdot \tau) \cdot \left(\frac{t_r}{t_{r(ref)}} \right)^{n_1} \cdot \left(\frac{C_r}{C_{r(ref)}} \right)^{n_2} \quad (7)$$

- $$SG = a + (b \cdot \tau + c \cdot \tau^2) \cdot \left(\frac{t_r}{t_{r(ref)}} \right)^{n_1} \cdot \left(\frac{C_r}{C_{r(ref)}} \right)^{n_2} \quad (8)$$

where is: ω –moisture content on the dry basis (kg_w/kg_{dm}); *SG* – solid gain (%), τ – time (min); t_r – solution temperature (°C); $t_{r(ref)}$ – referent solution temperature (°C); C_r – solution concentration (°C); $C_{r(ref)}$ – referent solution concentration (°C); *a, b, c* – function constants (-); *k* – drying constant (min^{-1}); n_1, n_2 – exponents of function (-).

Calculation of unknown constants, coefficients and exponents of the proposed mathematical models was performed by regression analysis that was done in the program package Statistica 10 (*StatSoft, Inc., 2010*). An assessment of the accuracy of the agreement of the tested models with the results of the experiment and the choice of the most suitable model were done through the correlation coefficient (R) as the basic criteria. And the statistical parameters were applied: chi - square (χ^2), mean bias error (*MBE*) and root mean square error (*RMSE*). Larger values of R accurate model and smaller values χ^2, *MBE i RMSE* indicate a greater precision of the model.

RESULTS AND DISCUSSION

Based on the results of dispersion analysis (Table 1 and 2) with the statistical probability of 99% can be concluded that there are statistically significant differences ($F > F_{crit}$) between the chosen levels of solution temperature and concentration compared to an average speed of drying nectarine halves and the increase of dry matter.

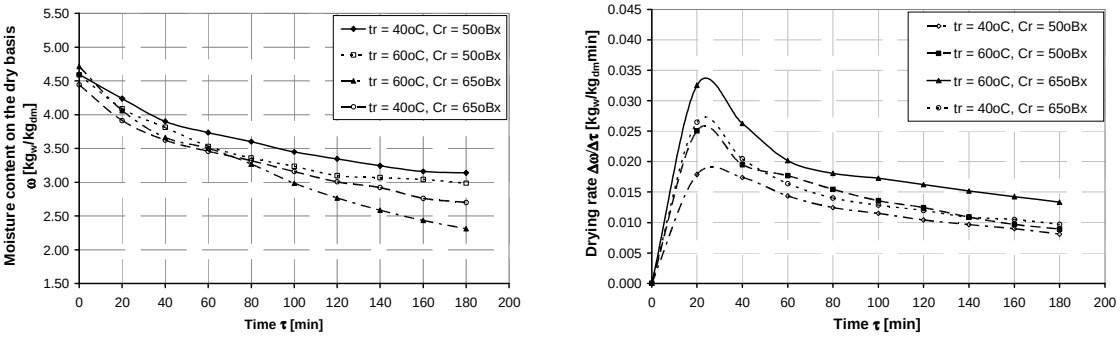
Table1. The results of dispersion analysis of the double-factor analysis for the statistical probability of 99% - Assessment of the influence of selected osmotic drying factors on the mean drying speed

Name	SS	DF	MS	F	p	F_{crit}
Intercept	0.001202	1	0.001202	1355.846	0.000000	6.918
Temperature of Solution	0.000015	1	0.000015	17.074	0.003289	6.918
Concentation of Solution	0.000027	1	0.000027	30.515	0.000558	6.918
Temp. and Concet. of Solution	0.000006	1	0.000006	6.863	0.030660	6.918
Error	0.000007	8	0.000001			

Table 2. The results of double-factor dispersion analysis for the statistical probability of 99% - Assessment of the influence of selected osmotic drying factors on the mean speed of solid gain

Name	SS	DF	MS	F	p	F_{crit}
Intercept	0.000011	1	0.000011	4004.469	0.0	6.918
Temperature of Solution	0.000000	1	0.000000	138.397	0.000002	6.918
Concentation of Solution	0.000000	1	0.000000	7.690	0.024180	6.918
Temp. and Concet. of Solution	0.000000	1	0.000000	5.099	0.053884	6.918
Error	0.000000	8	0.000000			

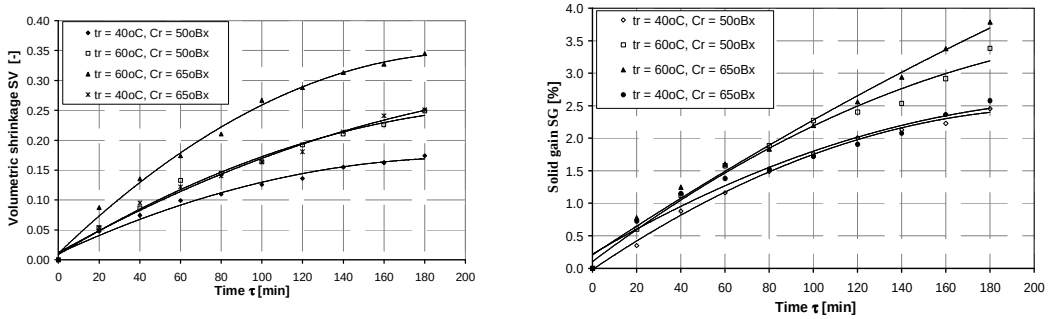
Figure 1a shows the kinetic curves of changes in mean moisture of nectarine halves with relation to dry base, and in figure 1b the mean drying rate compared to the dry base during osmosis.



a) b)

Figure 1. Kinetic curves of changes in mean moisture of nectarine halves with relation to dry base (a) and drying rate (b) during the osmotic drying time depending on temperature and concentration of osmotic solution

The largest decrease in moisture with relation to dry base was observed in the combination of temperature of osmotic solution at 60 °C and concentration of 65 °Bx, $\Delta\omega$ = 2.402 kg_w/kg_{sm}, and the smallest at the osmotic solution temperature of 40°C and concentration of 50°Bx, $\Delta\omega$ = 1.272 $kg_w/kg_{sm,}$ (Figure 1a). Drying rate increased intensively in the period of heating of the material at all combinations of temperature and solute concentration. After that, during the combining of factor levels t_r = 60°C and C_r = 65°Bx, mean drying rate is still significantly higher than the other combinations. For other combinations of factor levels, differences in mean speed of drying gradually reduce and equalize (Figure 1b). In Figure 2 increase in dry matter of nectarine halves is expressed in percentage with relation to the wet base(SG). Measured values (points in the diagram) are for the easier visual analysis of approximated second-order polynomial.



a) b)

Figure 2. Curves of mean increase in dry matter of nectarine halves with relation to wet base (a) and volumetric shrinkage (b) over time, depending on temperature and concentration of osmotic solution

The greatest increase in dry matter after 180 minutes in nectarine tissue was observed in combination of temperature of osmotic solution of 60℃ and concentration of 65°Bx, SG = 3.78 kg_{sm}/kg. The smallest increase of dry matter was observed in the combinations of osmotic solution concentration with lower values of temperature of osmotic solution, SG = 2,45 kg_{sm}/kg.

Figure 2b shows the results of volumetric shrinkage of nectarine halves during osmotic drying depending on the temperature and concentration of osmotic solution. The highest shrinkage was observed at osmotic solution temperature of 60°C and concentration 65°Bx, SV = 0.344. At osmotic solution temperature of 40°C and concentration 50°Bx recorded the least shrinkage SV = 0.174. The combination of solution temperature of 60°C and concentration of 50°Bx shrinkage is the same as during osmotic drying, where the temperature of the solution is 40°C and concentration 65°Bx SV = 0.25.

Influence of osmotic solution temperature factor is explained by high temperature solution lead to changes in the structure of cell membranes. The membrane becomes more permeable for water molecules, which diffuse through the tissue more intensively towards the interface, lowers the resistance of the tissue diffusion of moisture. Also, changing of the cell membrane partial permeability, solute molecules diffuse intensively from the osmotic solution into the inner layers of the fruit tissue. Higher concentrations of solution have resulted in the appearance of large concentration gradient of water and solute molecules, the interface between the fruit tissue and osmotic solution. The influence factors of osmotic drying are discussed in the same way by the authors: *Khoyi, and Hesari,* (2007) the apricot tissue in the form of thin sheets, *Park, et al.,* (2002) for osmotic drying of pear tissue in a cubic form.

Results of regression analysis of the kinetics of osmotic drying of nectarines in the studied form are given in Table 3.

Table 3. Results of regression analysis

Number of equation of mathematical model	Coefficients of the Model				Exponents of Model			*R*	χ^2	*MBE*	*RMSE*
	a	*b*	*c*	*k*	n_1	n_2	n_3				
(3)	4.106207	-	-	-0.002766	-0.078965	-0.280186	-	0.9527	0.03499	-0.000488	0.17748
(4)	18.8024	-	0.0169	0.0005	0.0558	22.8384	-	0.9440	0.04245	0.000986	0.19274
(5)	4.48006	-0.02152	0.00005		0.356201	1.006947	-	0.9886	0.00874	-4E-10	0.08762
(6)	0.093233	-	-		0.699048	0.694812	0.267643	0.9936	0.012907	-0.002209	0.10778
(7)	-	-	-	0.016718	0.785653	0.442887	-	0.9428	0.115586	0.100016	0.31802
(8)	0.11539	0.02322	-0.00002		-2.10990	-0.45632	-	0.9889	0.02285	3.85E-09	0.14141

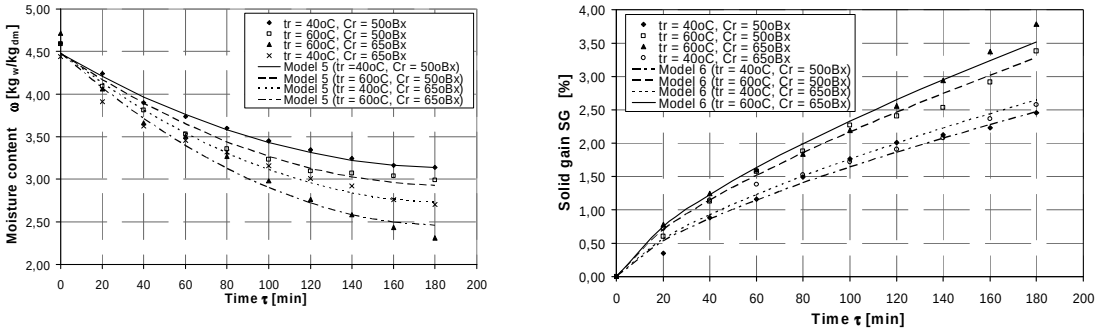
The highest coefficient of correlation (R), for mathematical models of moisture changes in relation to dry base during osmotic drying time has extended model *Wang & Singh* (equation 5). This model shows good agreement with the results of the experiment for the nectarine tissue, which is further confirmed by additional statistical indicators (χ^2*, MBE, RMSE).* The highest coefficient of correlation (R), for mathematical models of the kinetics of increase in dry matter compared to the initial mass during the osmotic drying time has extended *Degree model* (equation 6). This model shows good agreement with the results of the experiment for all three types of fruit tissue, which is further confirmed by additional statistical indicators (χ^2*, MBE, RMSE).*

When the final mathematical models for the change of moisture nectarine halves in relation to the dry base and increase in dry matter during osmotic drying are included, the calculated coefficients and exponents are:

$$\omega = 4{,}480 + \left(-0{,}0215\tau + 0{,}000057\tau^2\right)\cdot\left(\frac{t_r}{60}\right)^{0{,}3562}\cdot\left(\frac{C_r}{65}\right)^{1{,}0069} \quad (9)$$

$$SG = 0{,}0932\cdot\tau^{0{,}6990}\cdot\left(\frac{t_r}{60}\right)^{0{,}6948}\cdot\left(\frac{C_r}{65}\right)^{0{,}2676} \quad (10)$$

The derived mathematical models for nectarine tissue are applicable in a range of factors that they are derived for. Figure 3a shows the graphical comparison of changes in humidity of nectarine halves in relation to the dry base (ω) during osmotic drying time (τ) with the derived mathematical model 5, in figure 3b shows the graphical comparison of the increase in dry matter compared to the wet base (SG) during osmotic drying time (τ) with derived model 6.



a) b)

Figure 3. Comparison of measured values of change in moisture of nectarine halves in relation to dry base (a) with derived model 5 and increase of dry matter (b) with the derived model 6

CONCLUSION

Regression analysis of the measurement results of the osmotic drying experiment resulted in empirical models which describe the kinetics of osmotic drying of nectarine halves in sucrose. Two mathematical models were established, which describe: the change of moisture and increase in dry matter. The models are extended by influential factors in the dimensionless form. The derived models are with satisfactory correlation coefficients (R = 0.9886 and R = 0.9936) and as such may be useful in practice. The presented models are specific in their comprehensiveness. Using the derived model allows the calculation of the changes of modeled component over time in the range of factors in which the osmotic drying experiment is performed. At the same time, the models are sufficiently simple for the practical usage. With the help of standard computers it is possible to quickly calculate all modeled sizes, but also their application in control computers.

ACKNOWLEDGMENT

The results are a part of the project of the Ministry of Education and Science, the Republic of Serbia, No. TR – 03158, entitled “Combined Technology of Integrated and Organic Fruit and Vegetable Drying”.

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CAROB FLOUR ENRICHED GLUTEN FREE BREAD RHEOLOGY AND STRUCTURE

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ABSTRACT: In this study, gluten free breads (GFBs) made from rice and carob flour in different proportions were investigated. Water added changed in response to the carob amount. Structural and textural parameters of the samples such as viscoelastic properties of the dough, porosity, firmness, relative elasticity and microstrucure of crumb, were measured. Simple mathematical models were developed to correlate the measured properties with carob flour and water content. The simplest and most convenient mathematical model developed was a power model, indicating a significant water influence on GFBs characteristics . Carob flour addition enhanced the protein, fiber and minerals amount of the produced samples, and improved their functional properties as well, when water amount used was adequate. Rheological tests made, confirmed a shift towards a more elastic character when water content was low, typical for stiffer dough. .
Key words: *gluten-free, carob flour, structure, rheology, mathematical model*.

Nomenclature

A_o	surface of the compressed sample (cm^2)
a_1, b_1, a_{o1}, x_1, z_1 , a_2, b_2, a_{o2}, x_2, z_2, C_o, W_{to}	constants of the models (-), (-), (-), (-), (-), (-), (-), (-), (-), (-), (-), (%), (%)
C	percentage concentration of carob flour (%)
F,F_{max}, F_{res}	firmness of bread, maximum force for 25% compression of the crumb, residual force after 240s of relaxation phase (N)
h_o, h_t	initial and final height of the slice after compression (cm)
$REL\%$	relative elasticity of bread crumb
W_t	percentage amount of water (%)
ε, ε_s	bulk, surface porosity (%)
ε_o, F_o, Rel_o n_1, m_1, n_2, m_2, n_3, m_3	parameters which depend on the recipe of bread

INTRODUCTION

Celiac disease is considered to be a gastrointestinal disease, where genetically predisposed individuals exhibit intolerance in peptides released from wheat gluten. Cereals such as wheat, rye, barley, and probably oat are toxic for celiac patients. The only, up to now known treatment for celiac disease is strict adherence to gluten free diet. Rice flour can be a safe substitute of wheat for celiacs due to its bland taste, white colour, ease of digestion, and hypoallergenic properties (Gujral and Rosell, 2004). Furthermore gluten free products lack many important nutrients, such as proteins, vitamins, minerals and last but not least dietary fibres. (Lazaridou et al., 2007). For increasing the nutritional value of GFBs, pseudocereals like carob could be also used as protein source, enhancing also the overall nutritional value of the

gluten-free products, including their fiber amount. Carob germ flour can be used as dietetic human food (Dakia et al., 2007) or as a potential ingredient in cereal-derived foods for celiac people (Feillet & Roulland, 1998).
The objective of this study is the development of gluten-free bread formulations using carob and rice flour in different ratios changing also water content. Thus for a specific carob flour amount, water content has been changed in a broad range in order to find out the best combinations. Mathematical models correlating structural and textural characteristics of the final gluten-free breads to water/carob amount are developed and quantitative data are discussed. Micrographs were also taken using a Scanning Electron Microscope (SEM). Viscoelastic behavior of doughs was also evaluated performing frequency sweep tests.

MATERIALS AND METHODS

Materials

The materials used in this study were rice flour, moist yeast (L'hirondelle, S.I. Lesaffre, France), sugar, salt (iodised sea salt, Kallas, Greece), shortening (Vitam, Unilever S.A, Greece), egg white powder (Laffort S.A., Bordeaux, France), whey protein concentrate (Nutrilac®DR-7015, Arla Foods Ingredients Amba-Denmark) with 65% minimum protein content, emulsifier (DATEM:Diacetyl-tartaric esters of mono- and diglycerides, Danisco, Copenhagen), locust bean gum (LBG) (Sigma-Aldrich Chemie GmbH, Germany), enzyme of alpha-amylase with additional transglutaminase and hemicullolase activity (VERON CLX AB Enzymes, Darmstadt, Germany) and tap water. Moreover carob flour from the seed meal was partially integrated in the dough.

Bread making procedure

The basic dough preparation included the mixing of dry ingredients in a Hobart mixer (Hobart N50, Hobart Co., Troy, OH, USA) (carob flour, rice flour, sugar, salt, albumen powder, whey protein powder, Datem, LBG gum and enzyme) was followed by addition of melted shortening. The yeast was progressively mixed with the water amount and added to the final blend. The water amount added varied from 70-150% (on total flour basis) according to the carob flour content added. The water content range for each formulation was determined by conducting preliminary experiments, due to problems during farinograph measurements, in an attempt to find out the best combinations of water and carob content in gluten free breads. As it is known that dietary fibres addition absorb considerable amounts of water, it was seen that during these experiments carob flour formed a rigid structure that could not flow under gravity, requiring high amount of water to form bread with high quality, After complete mixing, the dough was fermented at 35^{o}C, 85% RH for 50 min. Following fermentation, samples were baked at 170^{o} C for 30 min in a convection oven. The loaves were cooled to room temperature and placed in polyethylene bags for 24 h before determination of their physical properties.

Bread analyses

Chemical analysis

Protein content was determined by Kjeldahl method for Nitrogen determination (ISO 937-1978). Total fat content was determined by the combination of acid hydrolysis with NaCl and the Soxhlet method using petroleum ether as a solvent according to Pearson's Chemical Analysis of Food (1981). The fiber content was determined using AOAC (1990) method 985.29. Total carbohydrates were determined by difference subtracting 100 g minus the sum of protein, dietary fiber, ash and fat expressed in g/100 g. The ash content was determined according to Pearson's

Chemical Analysis of Food (1981) and the NaCl content was determined according to Volharol method (Pearson's Chemical Analysis of Food, 1981).

Crumb texture

The firmness of bread's crumb was estimated with the 74-09 method of the American Association of Cereal Chemists (2000) in a Universal Testing Machine, equipped with a 50 N load cell. A slice of 2.5 cm (thickness) from the center of the loaf was compressed to 40% of its initial height with a 4 cm diameter probe coming down with a speed of 101mm/s.

The crumb's relative elasticity testing was carried out in crumb cubes of 2 x 2 x 2 cm (length x width x height). A uniaxial compression test with subsequent relaxation phase that lasted 4 min was applied in order to determine the textural and viscoelastic properties of the bread crumb. The relative elasticity of the crumb (the force with which the crumb resisted the defined mechanical stress during compression) was derived from the recorded force–time diagram. The calculation was done according to Equation 1:

$$REL\% = (F_{res}/F_{max}) \times 100 \qquad (1)$$

The measurements were done in triplicates.

Porosity determination

For porosity measurements, samples of 1.5 x 1.5 x 1.5 cm (length x width x height) of the geometric centre of the crumb were taken from all breads. The volume of solids (V_S, m^3) was measured with gas pycnometer (Stereopycnometer SPY-3, Quantachrome, Syosset, N.Y., USA) using helium as the displacement fluid for volume measurements. For each measurement three different samples were used, each measured three times.

SEM analysis

The structure of the crumb bread was analysed by scanning electron microscopy (SEM). Samples were freeze-dried and then mounted on metal stubs and sputter-coated with gold. Analysis of the samples was performed at an accelerating voltage of 10 kV with a SEM Jeol 6360 (Jeol USA Inc., Peabody, MA).

Dough rheology properties

The rheological properties of gluten-free dough were studied by a rotational rheometer (Rheometric Scientific SR5, United States) using a parallel plate geometry (mm diameter and 1 mm gap); the temperature was regulated at 25 ^oC by a RTE-130M circulating bath (Neslab, USA). The tests performed on the dough samples were: (a) strain sweep test in order to establish the viscoelastic region of dough samples and (b) dynamic oscillatory tests for a frequency sweep from 0.1 to 20 Hz at 0.5% strain. Each test was performed on different dough sample, at least in triplicates.

Mathematical Modelling

Various mathematical models were developed in order to determine the effect of the two independent parameters (the concentration of carob flour and water) in the total porosity, bread firmness and crumb's relative elasticity. The simplest and most convenient model according to the regression analysis was the power function.

RESULTS AND DISCUSSION

Chemical analysis

Table 1 shows the results of chemical analysis for breads with 15% carob content. Compared to a typical wheat bread this GFB has a high amount of dietary fiber and protein content; additionally it is a good source of microelements. According to Segura & Rosell (2011) the protein content of GFBs found in the Spanish market ranged from 0.91-15.05g/100g, with only one that contained soy protein had 15g/100g protein content. Thus, GFB with 15g carob flour/100 g flour can be considered a fibers' enriched product, rich also in proteins.

Table 1. Macronutrients and minerals amount of a gluten-free bread containing 15% carob flour

Macronutrients (g/100g bread)										
Protein		Dietary fibres		Fat		Ash		Carbohydrates		
8.37		6.10		2.04		1.76		38.70		
Minerals' concentration (mg/100g bread)										
Ca	K	Mg	Na	P	Al	B	Cu	Fe	Mn	Zn
75.93	112.02	51.72	381.89	99.58	1.01	0.15	0.19	1.65	0.69	1.17

Dough rheological characteristics

The viscoelastic region for the gluten-free dough formulations, measured by strain sweep experiments, was limited up to a strain of 1%; above this level, drop of elastic and loss modulus, G', G'' , respectively occur, indicating the breakdown of the gluten-free dough structure. Other researchers found 1% strain as the end of the viscoelastic region for gluten free formulations as well (Lazaridou et al., 2007; Sivaramakrishnan et al., 2004). Dynamic oscillation tests data concerning G' (f), G'' (f) and tan δ(f) for 5%, 10 and 15% carob content and for different water content are presented in Fig 1a, 1b, 1c, 1d.

All gluten-free dough formulations presented an elastic (or storage) modulus, G', greater than the viscous (or loss) modulus, G'' (f), in the frequency range. Tan δ values are always greater than 0.1 typical for weak gels. Concerning the impact of water content in dough formulations containing constant amount 5% of carob flour, the value of both moduli increased and that of tanδ decreased with a decrease in water content. This increase might be attributed to the fact that dough becomes stiffer since carob flour was not fully hydrated at low water content (Fig 1a, b).

The increase in carob content caused a shift of G' (f) and G''(f) towards higher values, while curve tan δ(f) exhibited lower values. Furthermore, frequency dependence on viscoelastic parameters was lower. The data indicate that carob addition caused an increase in bread dough elastic character e.g. led to a more pronounced solid elastic-like behaviour. Since both moduli increased with frequency, the gluten-free doughs exhibited a solid elastic-like behaviour. Therefore, tanδ (=G''/G') values for all dough formulations were lower than 1 (Fig 1 c,d). Similar observations on dynamic rheological studies have been previous reported for wheat dough supplemented with carob fiber (Mis´, 2011) and for rice flour dough (Lazaridou et al., 2007; Sivaramakrishnan et al., 2004).

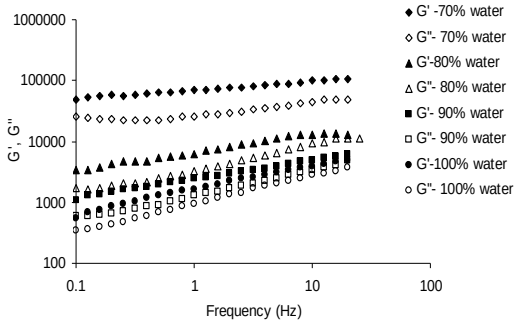

Figure 1a. G' (f), G'' (f) values for 5% carob content and different water content.

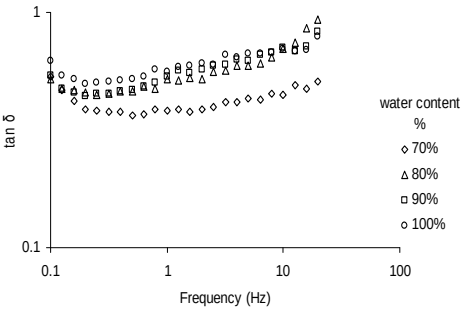

Figure 1b. tan δ(f) values for 5% carob content and different water content.

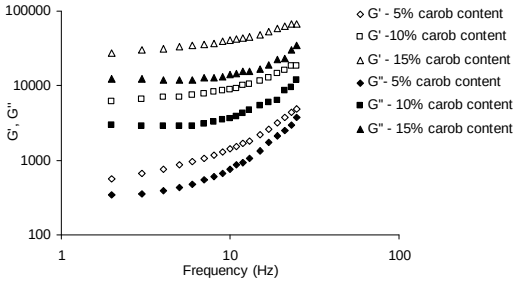

Figure 1c. G' (f), G'' (f) values for 100% water content for the three carob concentrations.

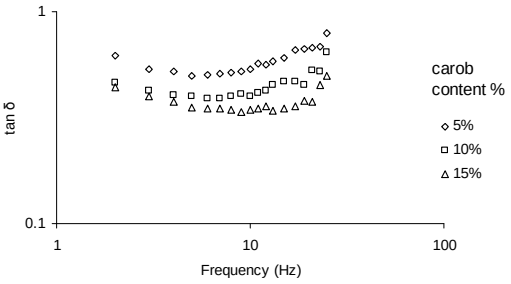

Figure 1d. tan δ(f) values for 100% water content for the three carob concentrations.

Crumb structural characteristics

Total porosity is influenced by both carob flour and water amount. As shown in Figure 2 and Table 2 porosity increases with increase in water but simultaneously is reduced with an increase in carob flour in an exponential relationship. However the influence of water is more pronounced that that of carob flour as shown by both figure 2 and mathematical model (Eq. 2 and Table 2). By changing carob amount from 5 to 15% in final GFBs, a reduction in maximum porosity values is observed that can be ascribed to fibers amount that may disrupt protein network and reduce bread volume.

Table 2. Mathematical model for Total porosity.

Total Porosity Model					
$\varepsilon=\varepsilon_o \cdot (C/C_o)^{n1} \cdot (W_t/W_{to})^{m1}$ (2)					
C_o	W_{to}	εo	n_1	m_1	R^2
10	100	44.41	-0.43	0.79	0.971

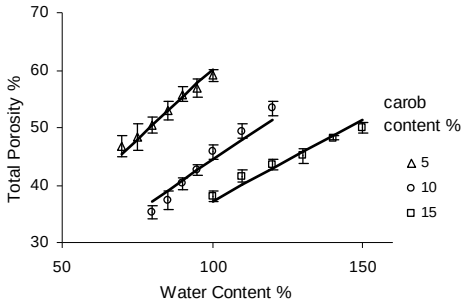

Figure 2. Influence of the carob and water content of GFBs on total porosity. Values are the averages of three replicates and error bars represent standard deviation. Solid lines represent theoretical values of the mathematical model (Eq 2).

SEM techniques were used to investigate the structural integrity of carob enriched gluten free bread's crumb. The addition of higher amounts of carob flour containing higher amounts of dietary fibers showed a large number of very small gas cells in an interrupted protein matrix, presenting a dense structure (Fig 3a. and 3b respectively). If the proportion of water is too low, the dough becomes brittle, not consistent leading to small or no development of the porous bread structure. In figures 3c and 3d GFB with 15% constant carob flour addition in different water contents is presented. In figure 3c with low amount of water content a coalescence of the gas cells may occur, evidenced by the small amount of gas cells presented. On the other hand sufficient water leads to a stabilized more aerated structure.

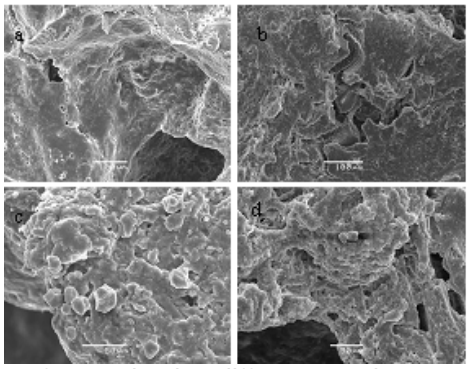

Figure 3. SEM micrographs of GFBs having different carob content /water ratios: 10/120 (a), 15/120 (b), 15/100 (c), 15/150 (d)

Texture analysis

Figure 4a shows that an increase in the water and carob flour content leads to a softening of the crumb. The greatest crumb firmness values were noticed in breads with 5% carob content (F_{max} = 48.7 N). Wang et al. (2002) indicate that the addition of carob fiber gives a softer bread crumb. However, firmness is mainly influenced by water content and in a much lesser extent by carob flour as shown in Table 3. Regarding relative elasticity, increasing the water there is a reduction in values observed (Fig. 4b). Carob flour can lead to a more elastic crumb as was found in the visoelastic tests of the dough

Table 3. Mathematical model for crumb's Firmness and Relative elasticity.

Firmness Model					
$F=F_o \cdot (C/C_o)^{n2} \cdot (W_t/W_{to})^{m2}$ (3)					
C_o	W_{to}	Fo	n_2	m_2	R^2
10	100	35.97	-0.00081	-1.20	0.917
Relative Elasticity Model					
$Rel\%=Rel_o \cdot (C/C_o)^{n3} \cdot (W_t/W_{to})^{m3}$ (4)					
C_o	W_{to}	Rel_o	n_3	m_3	R^2
10	100	0.74	0.30	-0.68	0.864

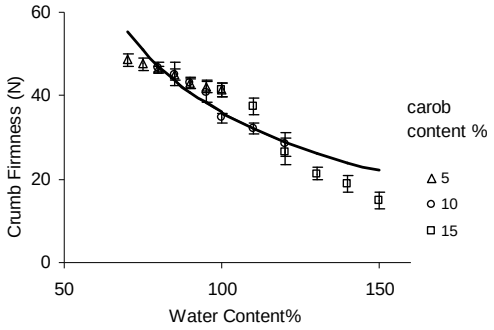

Figure 4a. Influence of the carob and water content of GFBs on crumb firmness. Values are the averages of three replicates and error bars represent standard deviation. Solid lines represent theoretical values of the mathematical model (Eq 3).

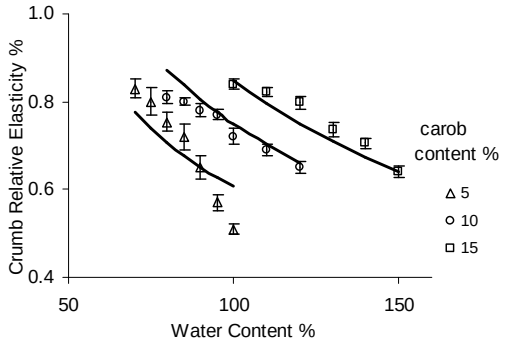

Figure 4b Influence of the carob and water content of GFBs on crumb relative elasticity. Values are the averages of three replicates and error bars represent standard deviation. Solid lines represent theoretical values of the mathematical model (Eq 4).

CONCLUSIONS

Based on the experimental results GFBs with carob flour / water ratio 10/110, 15/130 and 15/140, presented accepted quality parameters, with adequate dough proofing
crumb expansion and low firmness. The incorporation of carob flour as gluten substitute in breads results in bakery products with good functional attributes and improved nutritional value and can be proposed for a gluten-free diet.

ACKNOWLEDGEMENTS

This work was co-funded by the European Regional Development Fund and the Republic of Cyprus through the Research Promotion Foundation (Project ΥΓΕΙΑ/ΤΡΟΦΗ/0609(BIE)/08). The authors wish to thank the mills Kaplanides S.A. Serres- Greece for providing rice flour. We would like to thank the laboratory of Microscopy in AUA and specifically Dr. C. Fasseas for performing SEM analysis and for valuable discussions.

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THE OPTIMIZATION OF BEER PRODUCTION TECHNOLOGY BY ASSESSMENT OF MALTS PRODUCED BY VARIOUS EUROPEAN TERRITORIES, AIMING TO IMPROVE THE PROPERTIES OF BEER

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ABSTRACT: Given the overall demand for savings and quality improvement of beer, this study aimed optimizing of the production technology based on the evaluation of malts produced in Europe being reflected in the improvement of properties of beer in the factory "Birra Peja". The comprehensive study was carried out for the entire beer production chain, in close cooperation with the factory’s production and laboratory staff. The study was focused on malts produced in three European countries, Ukraine, Croatia and Serbia, as well as beer produced by these malts. These malts were analyzed in all stages of production, including chemical and microbiological analysis, as well as sensorial evaluation. Beer tasting for this study was done by two taster panels; panel of "Birra Peja" and the panel of Agricultural University of Tirana. We consulted with the working and laboratory staff at the "Union" brewery. Chemical and microbiological tests have been conducted based on European Beer Convention and MEBAK methods.Based on the conducted analysis and assessments of the beer quality, it was concluded that the beer produced with malts from Croatian origin, corresponds to a better quality of beer, which already is under production
Key words: Malt, Beer, production technology, EBC, MEBAK.

INTRODUCTION

The production of the beers was based on the a consistent formula at all stages of production, the same quantity and quality of water, the same amount of malt, the same generation *Saccharomyces carlsbergensis* type of yeast.
Starting from the first stage of beer production, one may observe various differences between the product and final product, such as saccharification time and rate, the apparent degree of fermentation, and the amount of polyphenols. An important factor is the amount of wort obtained at the end of the process or the degree of utilization of raw materials (malt) that is different from one type to another. Another very important factor is the presence of secondary products, such as high alcohols, which move from beer to beer.
In all cases of beer production technological parameters were the same, starting from the first stage in the domestication of the malt with water (quantity of malt, water quantity and water temperature).

MATERIAL AND METHODS

For this study, malts originating from Ukraine, Croatia, and Serbia are used. The first raw material used for the production of the beer: malts with the quantity of 4,500 kg, originally from Ukraine, Croatia and Serbia, water from the source of strong white Drin of 9ºDGH. In this case hops used on type originating from Slovenia Aurora (bitter) and Golding (aromatic) in report 70:30%. In all three cases there was 6.1gr α-acids/hl. Yeast which is used for production of these beers was *Saccharomyces carlsbergensis* with a concentration of about 20 million yeast cells per ml, or about 0.7 litres of a dense yeast hectoliter of wort. The processes and parameters in the phases of obtaining the wort were the same in all three cases.
The manufacturing process of the sweet solution was made with two decoctions. Cooling temperatures during the transfer to primary fermentation were the same (8 ℃ at the

beginning of the process). The temperature and pressure during the primary fermentation and complement was the same (8 ℃ - 15 ℃ and 0.5 b ar) for the three cases.
For the decomposition of β-glycan and the assistance of fermentation and decomposition of diacetyl enzymes used (α + β-glycan as amylase + protease, fungal-α-amylase, α-acetolactat decarboxylase).
At maturity, the beers stayed for 23 days at temperatures 0- (-1) ℃ and 0.5 bar pressure.
The beer was filtered with the same parameters and the same vehicle expense for filtering, (0.117030 kg / hl). The temperature was the same (25 min at 68 ℃) for pasteurization as well. At every stage of the production process of technological process is followed by microbiological tests which have been all time to level. Official methods for deriving the abovementioned results and analyzes are the following: the European Brewery Convention (EBC) and The Central European and Technical Analysis Commission "Methodensammlung der Mitteleeuropäischen Brautechnischen Analysenkommission" (MEBAK) .

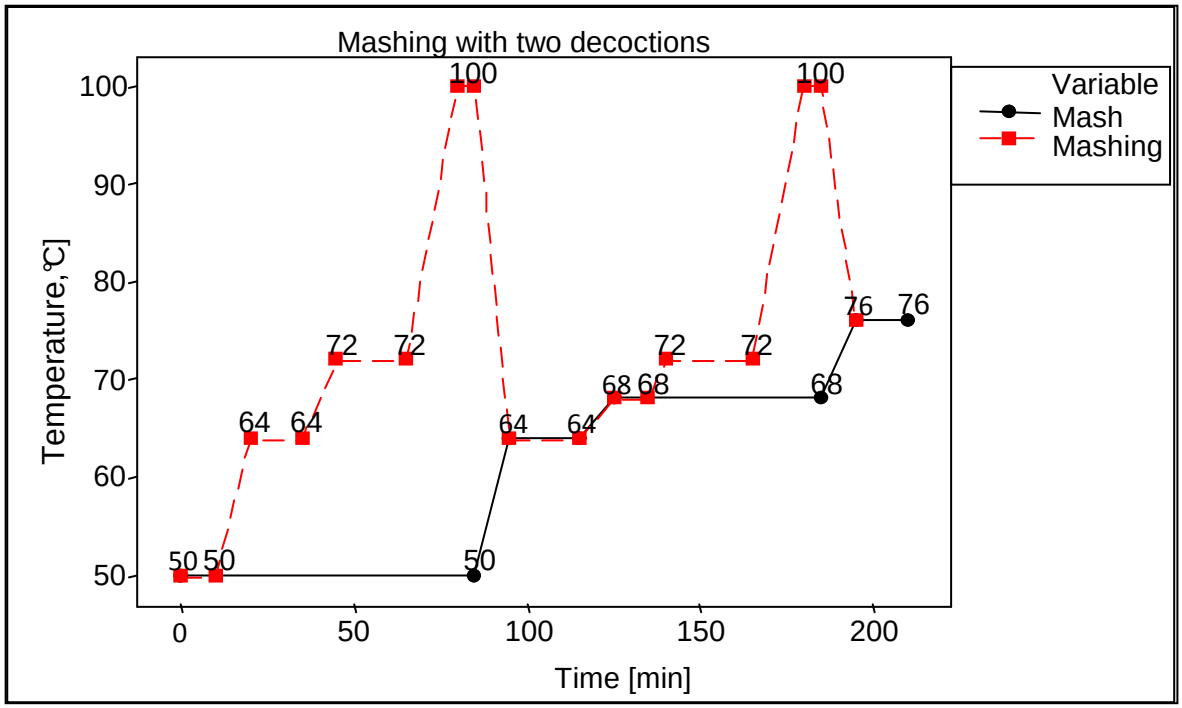

Figure 1. Ratio of mashing

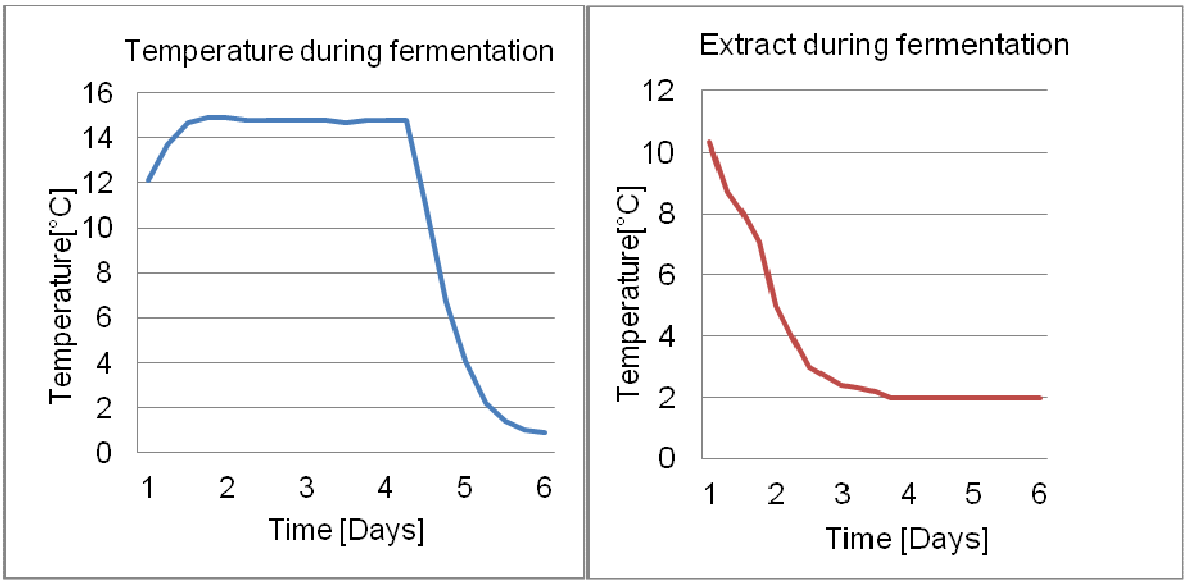

Figure 2. The temperature and extract levels during the fermentation process.

RESULTS AND DISCUSSION

Starting from the first stage of beer production, it may observes various differences between the product and final product, such as saccharification time and rate, the apparent degree of fermentation, and the amount of polyphenols. An important factor is the amount of wort obtained at the end of the process or the degree of utilization of raw materials (malt) that is different from one type to another. Another very important factor is the presence of secondary products, such as high alcohol, which move from beer to beer. The three cases have different degrees of fermentation as follows (1. 84.71 %; 2. 85.61 % and 3. 81.44 %).

	Beer made from malt			
	The quantity of wort	Extract of wort	Amount used of malt	Degree of utilization
	Hl, (hl)	E, (%)	G, (kg)	η, (%)
Ukraine	323.43	10.87	4500	78.30213527
Croatia	319.43	10.82	4500	76.9518811
Serbia	318.28	10.63	4500	75.37696873
	Report of the degree of utilization of raw material : 78.3% : 76.95% : 75.37%			

Figure 3. Degree of utilization of raw materials

Origin of malt	Ingredients	Hectolitre weight	Moisture	Extract dry weight	Sacchari-fication	pH	Color	Extract difference	Time of filtration	Hartong
	MEBAK I 4.1.3.1 max.0.5%	MEBAK I 4.1.3.3 48-62 kg	MEBAK I 4.1.4.1 max.4.5%	MEBAK I 4.1.4.2.2 min.81%	MEBAK I 4.1.4.2.4 max.15'	MEBAK I 4.1.4.2.7 5.7-5.9	MEBAK I 4.1.4.2.8.2 EBC 4-5	MEBAK I 4.1.4.2.10 max.1.7%	MEBAK I 4.1.4.11 max.40'	MEBAK I 4.1.4.11 37-42
Ukraine	0.49	0.49	4.3	80.3	9	5.91	4.0	1.1	27	40.64
Croatia	1.25	1.25	4.3	79.5	9	5.89	5.0	0.8	38	39.95
Serbia	1.04	1.04	4.3	77.1	8	5.96	3.8	0.6	45	36.72

Figure 4. Malt analysis used for study

Date of work	Basic extract %	Real extract %	Apparent extract %	Real rate of fermentation %	Apparent rate of fermentation %	Alcohol % v/v	Density 20/20	CO2 g/l	pH	Color EBC	Bitter EBC	O_2 total mg/l	Polyphenols mg/l
25.06.10	10.44	3.30	1.60	69.59	84.71	4.65	1.0062	5.0	4.53	7.0	22	0.20	145
10.07.10	10.40	3.20	1.49	70.30	85.61	4.67	1.0058	5.1	4.60	8.0	22	0.12	145
13.07.10	10.40	3.56	1.93	66.98	81.44	4.45	1.0075	5.2	4.57	8.2	21	0.17	152
min/max	10-11				78-84	3.7-4.7		4.7-5.7	4.2-4.6	7.5-10	20-26	0-0.5	145-175

Figure 5. Chemical analysis of beer produced during the study

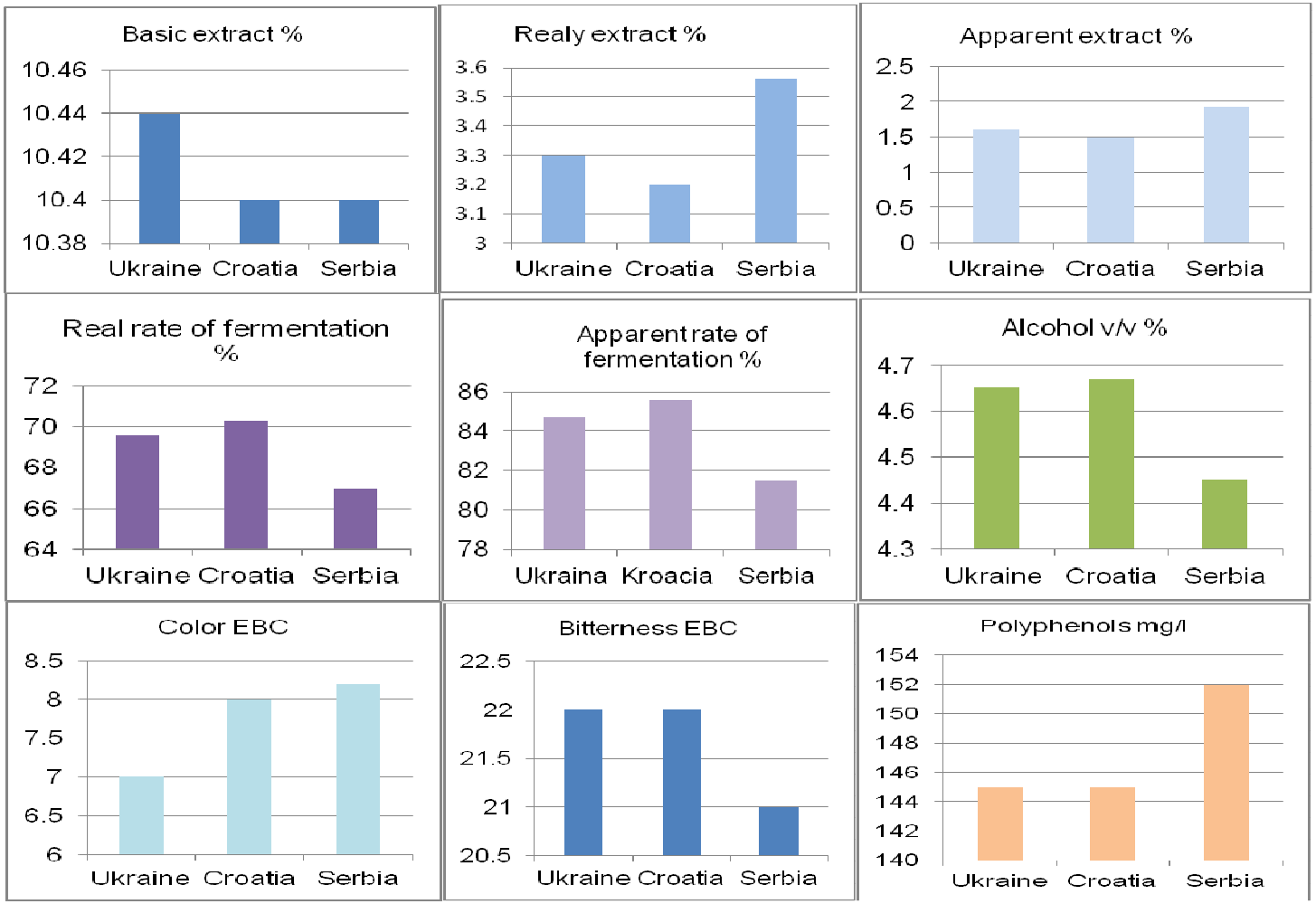

Figure 6. Histogram of chemical analysis of beers

Diacetyl (mg/l) 0.1	Penta-dion (mg/l) 0.6	Acet aldehyd (mg/l) 2-20	2-methyl sulphide (mg/l) 0.03-0.12	Ethyl acetate (mg/l) 5-30	2-methyl propanol (mg/l) 5-30	2-methyl butanol (mg/l) 5-20	Isoamyl acetat (mg/l) 1-5	Isoamyl alcohol (mg/l) 30-50
0.023	0.12	13	0.063	12	12	17	1,03	40
0.025	0.10	14	0.056	12	12	18	0,97	40
0.038	0.16	16	0.064	11	14	19	0,96	53

Figure 7. Secondary products

CONCLUSIONS

Based on work done in the period March 2010 - December 2010, has come to the conclusion that beer produced from malts originating from Croatia fulfills best conditions. On the basis of chemical and microbiological analysis beers, malts produced by Croatian origin were more qualitative compare to others. Beer stability is high; it is drinkable and shows the colloidal stability of high level.

These beers were evaluated by two tasting panels: panel test of the Agricultural University of Tirana as well as the panel test of JSC "Birra Peja". It is tested to Beer: taste, aroma, color, clarity, and foam. During these tasting beers that both groups have scored as follows: to taste (2.00; 2:45; 2.20), for flavor (1.57, 1:22, 1:35), for color (3.80, 4:45, 4:11) to light (3:42 ; 4:00; 4.00), and foam (3.10, 4.28, 4.44) Both panels evaluated with higher grade the beer produced from selected malts from Croatia.

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6. Prof.Dr. Vladimir Maric. *Biotehnologija i sirovine.*

EFFECT OF ELECTROMAGNETIC WAVES (MICROWAVE) ON STORAGE ABILITY OF YOLK LIQUID

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ABSTRACT:Microwave energy inactivates micro-organisms. In this study yolk liquid was pasteurized by using electromagnetic method in 3 different frequencies (2950-3950-4500 MHz) for constant time of 12 sec (6 sec of second pulse) and then results were compared with samples which were pasteurized at 65°C for 120 sec and control samples, after different times of storage(1,3,5,8,10 and 15 days). Results showed that used electromagnetic waves had significant effect on reduction of logarithmic count of aerobic mesophilic bacteria. According to results, however thermal pasteurization had a greater bactericide effect at zero time than cold pasteurization, but, a great increase was occurred in the number of aerobic mesophilic bacteria after passing time, in comparison with non- thermal pasteurized product. According to Iranian National Standard, in electromagnetic method liquid yolk shelf life has been defined 14 days which it is very ideal in comparison with thermal pasteurization (7d max) and control sample (2d max)
Key words: *electromagnetic waves (microwave), Yolk liquid, mesophilic bacteria, Non thermal methods.*

INTRODUCTION

Pasteurization inactivates some of undesirable enzymes; this method extends shelf life of liquid egg yolk around 7 days, 10 days, 14 days and even 16 days. It depends on relation between thermal processing temperature and length of time (Schlegel, 1992). Pasteurization is defined as “a process of heating food for the purpose of killing harmful organisms such as bacteria, viruses, protozoa, molds, and yeasts.” (Lewis and Heppell, 2000). A process has been developed for the non-thermal treatment of liquid food products which results in a significant reduction in the microbial population, thus reducing spoilage and extending shelf life. In egg yolk because of low pH and higher solids content, Egg yolk is an excellent media for the growth and multiplication of bacteria and bacteria are able to grow faster than whole egg and albumen, so egg yolk pasteurization temperature must be higher than pasteurization temperature for egg and albumen (Stadelman *et al.,* 1995). Proteins are highly heat sensitive components of the egg. The functional properties like whip ability, foam ability, foam stability etc. which make the eggs an inevitable ingredient of various food products are severely affected by high temperatures. Also experimentally it is found that the egg yolk needs to be heated to a higher temperature than the albumen (Dev *et al.,* 2008). Since yolk pasteurization changes the amount of solid substances, it is effect on stability and resistance of the emulsifier, also in consequence of thermal process, proteins will be denaturised and formed disulfide bonds that will cause to release sulphur gas and make a bad smell (Hanson *et al.,* 1946). Heating leads to death in foodstuff bacteria, either it is effect on aroma, flavor, texture and nutritional value of processed food. Among different methods of antisepticising of food, because of protection nutritional value of product, improve the quality of food and decrease of its cost that it is important for customer the non -thermal methods which results in significant reduction in microbial count, and decreasing of spoilage and increasing shelf life of food products these methods have been received more attentions (Hasanzade, 2008;

Huang, 1989). This study examined the effect of cold pasteurization by electromagnetic waves (microwave) on liquid egg yolk and reduction of logarithmic amount of present aerobic mesophilic micro-organisms in samples, and compared them with current thermal methods (regular pasteurization).

MATERIAL AND METHODS

Sample preparation

Samples were selected from a fresh and healthy batch of egg and it was broken by egg breaker equipment, and was separated (yolk and white egg), was filtered (to separate crust and small particles) and was homogenized. These was performed by selecting fifty 100 g samples from 100kg liquid yolk randomly and they were filled in special poly ethylene bags automatically after filtering and they were stored at 0 to 4ºC until experiments.

Instrumentals specifications

Electromagnetic pasteurizer was used (model SCP150, Afra Sanat Kimia Mashhad co, Mashhad, Iran). This device was able to work in variety of temperature, times and frequencies.

Method

Four Samples were selected randomly, then, one of them was retained as control sample and other three samples were treated under 3 frequencies (2950, 3950 and 4500 MHz). Each experiment replicated 10 times. Prepared packaged samples placed in the device chamber and it was set turn on and turn off magnetrons at specific times. It was considered 2 pulses, 6 sec ON and 8 sec OFF it means that the total exposure period was defined 12 sec and total procedure period was 20 sec. The storage time was defined accordingly to avoid of egg yolk coagulation that it is the maximum time which 100 g liquid egg yolk could expose to electromagnetic waves at max 40 ºC without any damages or changes in its physical properties in comparison with fresh (control) sample. After setting pulses and time switch the magnetron1 (frequency 1 ON and turn the system ON, and has taken out the sample, after the set time has finished and system turned OFF, second and third samples exposure to higher frequency by defined method respectively, then all samples were treated under the same pasteurization method. In this method temperature of product center was below 40°C. Also egg yolk liquid samples were pasteurized at 65°C by regular pasteurizer and then automatically filled in airproof packages by filler and placed in refrigeration room. Then samples transferred to laboratory for cultivation with culture medium of plate count agar in 2 repeat, and cultivated in 30 °C for 72 hours according to national standard NO. 5272, and Total Aerobic Mesophilic Count (TAMC) were determined. Results were compared with those which they are as control and thermal treated samples. After that treated samples which stored in suitable condition (0°C to 4°C) for 1, 3, 5, 8, 10, 15 days for studying the effect of storage time on TAMC, then cultivated and TAMC was determined again. Results were compared with permitted total count of mesophilic bacteria's in pasteurized egg, with consider to Iranian national standard (13248) (Institute of Iran Standard and Industrial Researches, 2007; Institute of Iran Standard and Industrial Researches, 2010)

Statistical analysis

Each treatment was analyzed as randomized complete block design with ten replications and the data were assessed by analysis of variance (ANOVA) and Duncan s multiple range test using MSTAT-C software program. Differences among treatments were tested with least significant difference (LSD) test ($P<0.05$). Besides, correlation analyses were performed to clarify the relations among parameters considered in this study. Microsoft Excel 2007 was used to plot apparent.

RESULTS AND DISCUSSION

Effect of different electromagnetic frequencies (microwave) and different storage time on logarithmic count of aerobic mesophilic bacteria

Results showed that pasteurization by electromagnetic waves had reduced count of aerobic mesophilic bacteria in egg yolk liquid at least 0.30 to 0.46 logarithmic cycles instantly after cold pasteurization in comparison with control samples. Microwaves have the ability to generate heat from within the substance that is exposed to it. The shell egg appears ideally suited for pasteurization in a microwave environment (Fleischman, 2004; Rehkopf, 2005). The results confirmed with results of kozempel et al. (1999) which reported microwave energy has a significant effect on reduction of microbial population in egg yolk liquid (Kozempel *et al.,* 1999). Results also agreed with results of Dev et al. (2008) which showed, in this study, microwave heating has been considered for in-shell egg pasteurization. First of all, the effects of temperature (0-62^{o}C) and frequency (200 MHz to 10 GHz) on the dielectric properties of egg components were investigated. Laboratory trials on microwave heating of in-shell eggs indicated that the heating rates of both albumen and yolk were similar. Therefore, microwave heating appeared perfectly suited for in-shell egg pasteurization (Dev *et al.,* 2008). According to results, that obtained from statistical analyzes, effect of adding frequency variants ($P<0.05$) and pasteurized product durability ($P<0.01$) on reduction of total aerobic mesophilic count in liquid egg yolk in α level was 0.05 (95% probability) and it was statistically significant, but no interaction was observed between studied variants in cold pasteurization process. Storage time which was studied up to 15 days has a higher effect on TAMC than various frequencies applied so the value of increasing TAMC was higher than reduction of it during yolk liquid pasteurization process by different electromagnetic waves. Comparing averages of three types frequencies used in liquid egg yolk cold pasteurization and reduction of TAMC, experiments showed that killing ability of waves has decreased by increasing frequency from 2950 MHz to 3950 MHz, and then by increasing frequency to 4500 MHz efficiency of electromagnetic method in reduction of microbial population in pasteurized product significantly increased (figure. 1). As shown in figure 1. Minimum TAMC was in samples which were treated by frequency 4500 MHz and it was 3.68 cfu/mL, that a reduction was found around 1.2 logarithmic cycles in comparison with control samples. The least lethal effect was at 3950 MHz that it has a decline around 0.97 logarithmic cycles comparing with control sample. Lethal ability of microbial population at 2950 MHz frequency was medium, however it was better than 3950 MHz frequency (by reduction around 1.1 logarithmic cycles) but it was not statistically significant.

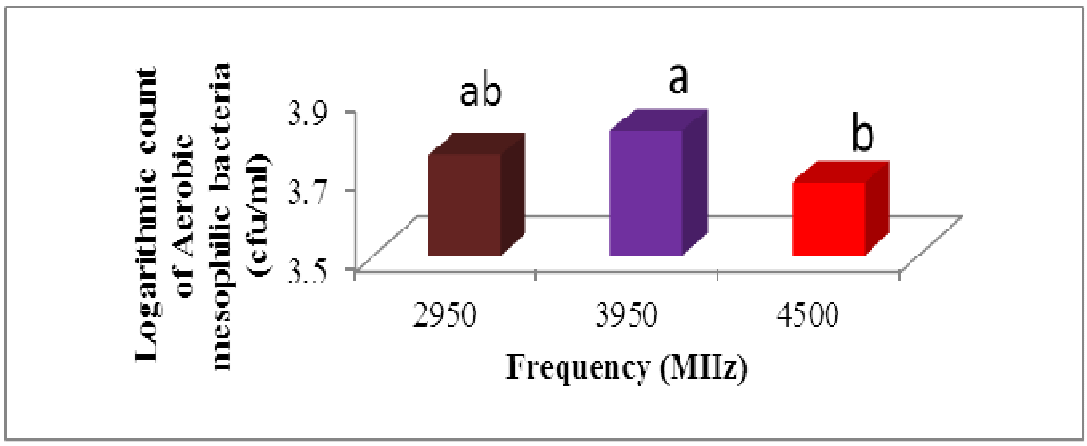

Figure 1.Effects of different frequencies on TAMC in liquid egg yolk cold pasteurization procedure

Therefore it has been confirmed that in a way of better functionality and higher lethal ability frequencies are in this order: 3950 MHz <2950 MHz <4500 MHz but 3950 was an exception which it has a lower killing effects. Results show that by increasing storage time of pasteurized product from 0 days to 15 days, TAMC rise rapidly, depend on intensity of

treatments, increased by different growth rates (Figure 2.). TAMC immediately after pasteurization at 4500 MHz was 3.17 cfu/mL and went up to 4.38 cfu/mL in 15th day. TAMC instantly after pasteurization (zero time) at 2950 MHz and 3950 MHz were 3.27 cfu/mL and 3.33 cfu/mL, respectively while in 15th day it reached 4.42 cfu/mL and 4.50 cfu/mL respectively. This agreed with Sevcan et.al. Studies on variation in dielectric content of fresh egg in storage period and relation of it with egg freshness and either confirming that ε' (dielectric properties rate) and ε" (dielectric lose factor rate) in egg yolk, will increase during storage (Unluturk *et al.,*2008).

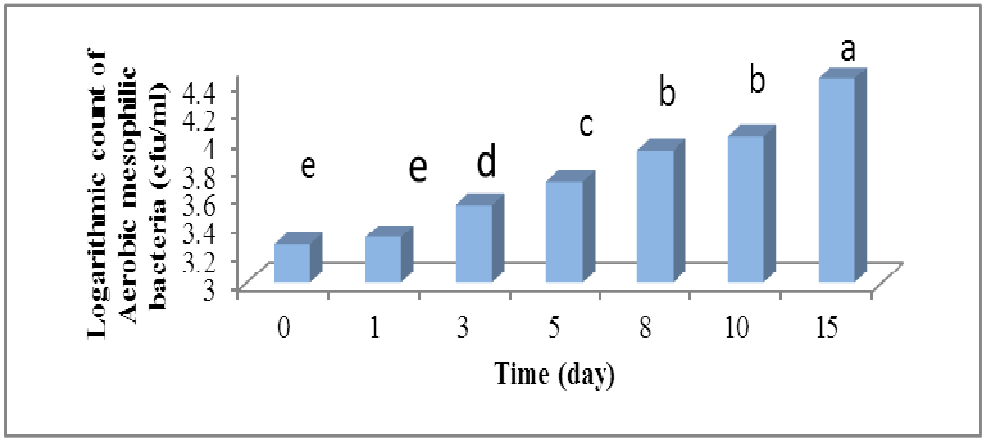

Figure 2. Effect of different storage time on logarithmic amount of aerobic mesophilic bacteria's in yolk liquid cold pasteurization procedure

Comparison between liquid egg yolk cold pasteurization, electromagnetic method and pasteurization by thermal treatment

Results showed that in zero time (immediately after treatment), thermal pasteurization can decline TAMC around 0.52 and pasteurization by electromagnetic method can decrease TAMC nearly 0.3 to 0.46 logarithmic cycle comparing with initial control samples (Table 1). Thermal pasteurization method was more effective in destroying bacteria in comparison with cold pasteurization in zero time, but because of high advantages of non -thermal methods, this difference is connive. Therefore, results of this study, confirmed different studies, include Goldblith and Wang (1967), Mudgett (1982) and Fujikawa et al. (1992) welt et al. (1993) showed that there isn't a significant difference between microwave heating and regular heating (Goldblith and Wang 1967, Mudgett 1982, Fujikawa *et al.,* 1992, welt *et al.*, 1993). Results generally showed that both treatment, include thermal and electromagnetic have significantly decreased microbial amount in comparison with blank samples, and this difference was observed during product storage days. According to results, cold pasteurization by electromagnetic waves decreased mesophilic bacteria population more than regular methods, especially during storage days, product which had been treated by waves, had less increasing microbial amount (Figure 3.). As it could be observed, even worst electromagnetic treatment (in 3950 MHz) has better worked in comparison with thermal treatment (thermal treatment<3950 MHz<2950 MHz<4500 MHz). Results of this research showed that usage of both heat and waves could have stronger effect on lethal factor and it confirmed results of Kozmpel et al. which demonstrated that microwave energy may complete or strength thermal effects (Kozmpel *et al.,* 1999). Sanvo co. (2010) used microwave method combined with 65°C heating and claimed that product heated to only coagulation and coacolation point and consequently the product was completely like fresh egg. This process decreases total amount of bacteria 10 times more than regular pasteurization methods. Studies showed that the method will protect egg functional properties 20% more than regular methods (Colavitti *et al.,* 2010). There is significant reduction in process time which is important in results of other studies, also in this study confirmed total time of process is only 20 sec that it is very short comparing with 120 sec time of thermal pasteurization. In comparison with regular methods for heating foodstuffs,

pasteurization by microwave potentially could improve organoleptic properties, appearance and nutritional value. During storage time minimum amount of aerobic mesophilic bacteria was determined in samples treated by waves and since first day it was observed that increased amount of aerobic mesophlic bacteria in thermal treatment. It was much higher than treatments by waves, for example in 4500 MHz frequency logarithmic number of bacteria increased from 3.17 at the first moment after process to 4.38 in 15th day, (i.e it has increased 1.21 logarithmic cycle), whereas in thermal treatment from 3.11 immediately after process reached to 4.99 in 15th day (i.e. it has increased 1,88 logarithmic cycle). As it has shown in Figure 3. Logarithmic number of bacteria in 4500 frequency which was equal to 3.68 cfu/mL during storage days as it showed reduction around 1.2 logarithmic cycles in comparison with control sample. Minimum killing effect was in 3950 MHz which had a reduction around 0.97 logarithmic cycles. Killing effect of 2950 MHz was halfway and it was almost 1.1 logarithmic cycles and in thermal treated samples, total aerobic mesophilic bacteria was equal to 4 cfu/mL and killing effect was around 0.74 logarithmic cycle. Results showed that, however thermal pasteurization was better on destroying bacteria than cold pasteurization at the first time, microbial amount increase more rapidly during storage time.

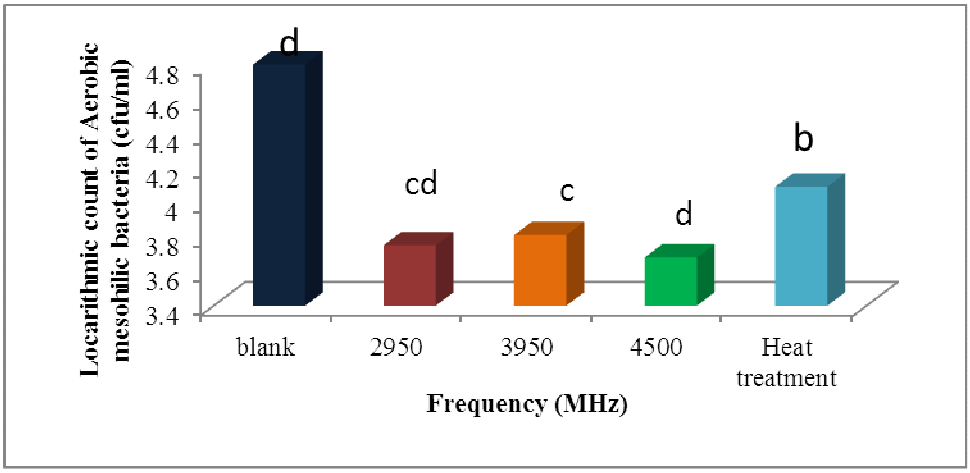

Figure 3. Comparison of yolk liquid cold pasteurization procedure using different frequencies with blank samples and thermal pasteurization procedure

Table1. Average aerobic mesophilic bacteria's population, under different treatments and different storage days

Blank	Heat treatment	4500MHz	3950MHz	2950MHz	Storage day
3.63	3. 11	3.17	3.33	3.27	0
4. 3	3. 55	3.25	3.38	3.34	1
4. 72	3. 75	3.49	3.59	3.55	2
4. 92	4. 12	3.66	3.77	3.71	3
5 .07	4. 45	3.87	4	3.93	8
5. 24	4. 69	3.97	4.10	4	10
5. 96	4.99	4.38	4.5	4.42	15

Determination pasteurized sample's storage time according to Iranian national standard limit

Microwave energy inactivates micro-organisms through thermal kill. It also has the potential to cause biological damage as well as alteration of the cell membrane and metabolic functions. (Huang, 1989). Maximum authorized logarithmic amount of liquid egg pasteurized mesophilic bacteria at 0°C – 4°C is 4.47cfu/mL and it was defined 30000 pcs/mL according to Iranian national standard. At the end of third day, results showed that logarithmic amount of mesophilic bacteria in control samples (non- pasteurized) is 4.72 cfu/mL (Table 1). Therefore, maximum storage time of non-pasteurized liquid egg is two days. At the end of 8th day, results confirmed that total aerobic mesophilic count in thermal treated samples is

4.45 cfu/mL (Table 1). Therefore, maximum storage time of liquid egg pasteurizing by regular thermal method is 7 days. At the end of 15th day results showed that number of aerobic mesophilic bacteria in cold pasteurized samples is 4.38- 4.5 cfu/mL (Table 1). Therefore, maximum storage time of liquid egg which pasteurized by waves method is 14 days. Finally it was observed that the rate of increasing total aerobic mesophilic count in product that was pasteurized by this method (cold pasteurization in electromagnetic method) was much slower than regular thermal method. Tang et al. (2002) by similar studies on bean showed that the product processed by microwave had better color and taste than those pasteurizing by regular method in cans and shelf life of sterile product by microwave will increase (Tang *et al.,* 2002). Consequently, shelf life of pasteurized liquid egg yolk in electromagnetic method defined maximum 14 days which is more ideal comparing with shelf life of it in regular methods, 7 days, and control sample (non-pasteurized yolk liquid), 2 days.

CONCLUSIONS

Liquid egg yolk cold pasteurization significantly decreased total aerobic mesophilic count of samples. Therefore this method can be used in liquid egg yolk manufacturing industry. Meanwhile present obstacles in use of pasteurized yolk liquid in the country (because of changes in quality and functional properties comparing with fresh egg) will be eliminated and like other modern countries in the world, national legal organizations can persist on obligation of using pasteurized liquid egg in egg consumer units. Because raw egg consumption that includes a huge risk, will reduce in products such as mayonnaise sauce and therefore could effect on the usage of chemical preservative substances in these products. Also it was observed that product shelf life in this method has increased two times comparing with regular thermal method. It shows that in spite of initial amount of vegetative bacteria in regular thermal method was lower at the first time, it wasn't able to terminate bacteria spores equal to wave method, that this is very important for consumers and makes higher assurance for shopping and storage of product.

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SHELF LIFE EXTENSION BY HIGH PRESSURE OF A VEGETABLE BASED MEDITERRANEAN DELI SALAD

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ABSTRACT: With the objective to replace mayonnaise as the basis of a ready-to-eat traditional Greek deli salad, corn, carrots, cucumbers, peas, capers and Aloe Vera and lemon juices were combined into an emulsion like homogenized avocado puree. All ingredients were of Greek organic agriculture. The product was vacuum packaged in transparent, polypropylene containers. The objective was to achieve a commercially viable product fresh-like quality and extended shelf life. High Pressure (HP) processing, targeting on microorganisms and enzymes inactivation while maintaining the sensorial quality of the food was used. After refining the formula of the product for final texture, flavour and quality, it was HP processed at pressures ranging from 100-800MPa and process times from 2 to 30min to allow for optimal HP conditions selection. The main quality deterioration factor for avocado-based products is the enzymatic browning attributed to polyphenoloxidase (PPO). Control of PPO activity is prerequisite for product stability through time. Based on sensory evaluation and colour degradation of the processed samples, the conditions selected were 600 MPa for 15 min. Adequate microbial and enzyme inactivation was measured at these conditions, while texture and flavour were maintained. Shelf-life experiments were conducted at temperatures from 0 to 15 ºC. Based on the quality indices monitored and modeled with time, the shelf life of this product was estimated as 72, 45, 20 and 7 days for storage at 0, 5, 10 and 15 ºC, respectively.
Key words: *High pressure, extended shelf-life, avocado-based products, stability*

INTRODUCTION

Several traditional Greek deli salads are based on a mayonnaise emulsion with a variety of ingredients mixed in, including vegetables such as eggplant, carrots, potatoes, traditional pasta components and herbs and spices. The aim of food producers is to extend the line of such products, strengthening the healthy image, by replacing the mayonnaise basis with a no egg, no added fat vegetable based matrix. Avocado puree, rich in nutrients, and unsaturated fats could be used as a substitute of mayonnaise. However, a major fraction of avocado protein content has been reported to be composed of oxidative enzymes such as polyphenol oxidase (PPO) (Kahn 1977; Elez-Martinez et al, 2005). When avocado pulp is processed from the intact fruit, the tissue undergoes partial disruption of cellular organelles releasing PPO enzyme that reacts with its substrates (phenolic compounds), causing the formation of *o*-quinones. These compounds undergo further polymerization producing brown pigments, undesirable flavors, and nutritional losses (Weemaes et al, 1999; Tomas- Barberan and Espın 2001). Consequently, the main quality deterioration factor for avocado-based products is the enzymatic browning attributed to PPO. Control of PPO activity is prerequisite for product stability through time.

Conventional thermal processing (thermal pasteurization) could be used to inactivate PPO from avocado products, but the temperature has negative effect on sensory (development of bitter taste) and nutritional characteristics. On the other hand, High pressure (HP) could be used in commercial-scale operations as a non thermal processing technology in order to inactivate the spoilage microorganisms and endogenous enzymes (Lopez-Malo et al,1998;

Palou et al,1999;2000; Katsaros et al 2010) as well as to extend the shelf-life without affecting their quality characteristics. Application of HP processing on acidified avocado puree (addition of lemon juice) could minimize the detrimental quality modifications induced by residual PPO activity and assure product safety. The objective of this work was to study the quality and shelf-life extension of a commercially viable non mayonnaise based, vegetable (avocado based) deli salad.

MATERIAL AND METHODS

Product formula refining

The developed 'Green Mediterranean Deli Salad' composition is similar to the widely known Russian salad, substituting the mayonnaise with avocado puree and adding some health promoting ingredients. The product composition can be characterized by two parts; the avocado base and the rest vegetable based ingredients:

Base: Avocado puree (54%) was used as a base for the production of this salad. Avocadoes are rich in nutrients such as dietary fibres, vitamin B6, vitamin C, folates, vitamin E, potassium and magnesium. They have a mild fresh flavour and the texture of their puree can be similar to the mayonnaise emulsions. *Aloe Vera* juice (3%) was added to avocado puree targeting in the desired textural characteristics of the final product, while simultaneously strengthening its nutritional-functional characteristics.

Vegetables: Whole vegetables or vegetables cut in cubes were added to the base (avocado puree) adding nutritional value to the product. The addition of different vegetables of different colours made the final product more attractive to the consumers and contributed to the desired flavour and taste. The ingredients - all products of Greek organic cultivation - that were added for the formula refining are: corn (11%), cucumber (9%), carrot (8%), caper (3%), peas (2%), salt, pepper and lemon juice for pH adjustment equal to 4.

A number of steps using appropriate equipment that consists of vegetable processing units, packaging equipment, High Pressure Unit for cold pasteurization and cold chambers for the storage of products are necessary for the final product production (Figure 1).

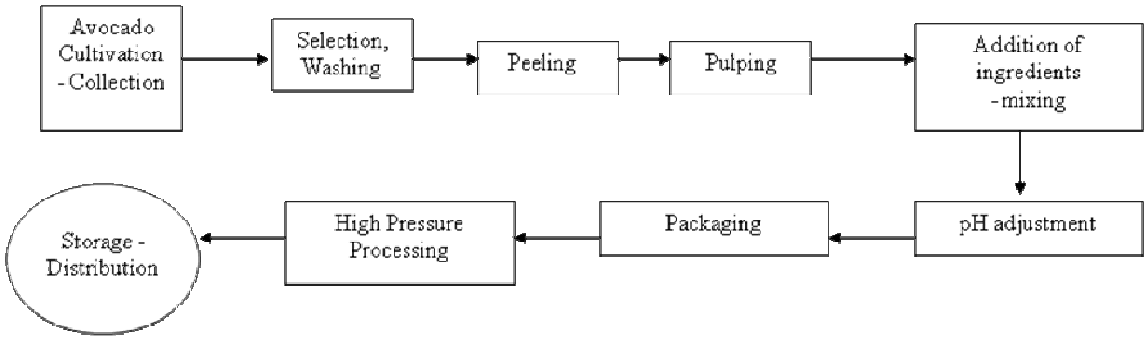

Figure1. Flow chart for the production of Green Mediterranean Deli Salad

For the experiments, samples (200 g) were packed into multilayered bags (PP-aluminium leaf-PE) for HP processing. High pressure treatments were achieved using a 1.5 L volume HHP equipment with a maximum operating pressure of 1000 MPa and temperature control (Food Pressure Unit FPU 1.01, Resato International BV, Roden, Holland) by a liquid circulating jacket connected to a heating-cooling system. Pressure was released after preset time interval by opening the pressure valve. Pressure and temperature were constantly monitored and recorded (in 1 s intervals) during the process. HP processed and untreated samples were stored at 0, 5, 10 and 15 ºC for over a period of 72 days. Selected quality indices such as sensory evaluation, microbiological analysis and colour were measured through time for the determination of Green Mediterranean Deli Salad shelf-life. Evaluation of the selected quality indices for all the samples was performed at specific time intervals. The shelf-life determining factors were browning and bitter taste.

Polyphenoloxidase activity
The PPO activity was measured as described by Pizzocaro et al. (1993). For HP treated and untreated samples, 10 g were mixed and homogenized with 10 ml of citric-phosphate buffer solution (pH 6.5). The homogenate was centrifuged (4000xg) at 4 ºC for 30 min and the supernatant was filtered and analyzed for PPO activity at 420 nm and 25 ºC. One milliliter of catechol solution (0.175 M) and 2 ml of buffer solution pH 6.5 were added to 0.5 ml of PPO extract. PPO activity was assayed in triplicate in a Spectrophotometer (Helios Unicam, USA) and calculated on the basis of the slope from the linear portion of the curve plotted with ΔA_{420} versus time up to 2 min for both fresh and HP treated avocado purees. One unit of PPO activity was defined as 0.001 ΔA_{420} min^{-1} (ml of extract)$^{-1}$. Residual PPO activity was expressed as the ratio between treated and fresh avocado purees.

Color measurement of avocado-based salad
Quantification of the colour change was based on measurement of CIELab values with a CR-200 Minolta Chromatometer (Minolta Co., Chuo-Ku, Osaka, Japan) with an 8 mm measuring area. At predetermined times of isothermal storage, according to the design, measurements were conducted and values of ΔC and ΔE were determined.

Microbiological analysis
Microbiological analysis was conducted in HP-treated and untreated samples of Green Mediterranean Deli Salad. Using the spread-plate technique, total plate count (PCA) and yeasts and moulds (RBC) were measured in all samples after incubation at 25 ºC for 72h and 120h, respectively. All received data were estimated as $\log_{10}$ values.

Sensory evaluation
For the sensory evaluation all the samples were removed from the storage cabinets and stored at 15 ºC until temperature equilibrium (approximately 30 min). The colour, taste and general impression of each sample were scored by a 9-member organoleptic panel, using a nine-grade hedonic scale. The value equal to 5 was set as the acceptance/non-acceptance limit.

RESULTS AND DISCUSSION

After refining the formula, the final product was HP processed at pressures ranging from 400-600 MPa and process times from 5 to 15 min at 20℃, to allow for optimal HP conditions selection. Based on minimum sensory and colour degradation and microbial (total microflora) and enzyme (PPO) inactivation, the process conditions selected were 600 MPa for 15 min. Figure 2 presents the residual PPO activities for the studied high pressure treatments. For treatment at 600 MPa and process times 5, 10 and 15 min, the residual PPO activity in avocado puree was estimated as 68, 36 and 5% of the initial activity, respectively. Pressure treatment of the avocado puree at a pH value 4, significantly affects its PPO activity. This is in agreement with the study of Eshtiaghi and Knorr (1993) who reported that reducing the pH of the immersion medium by addition of citric acid combined with high pressure treatments could lead to increased PPO inactivation.

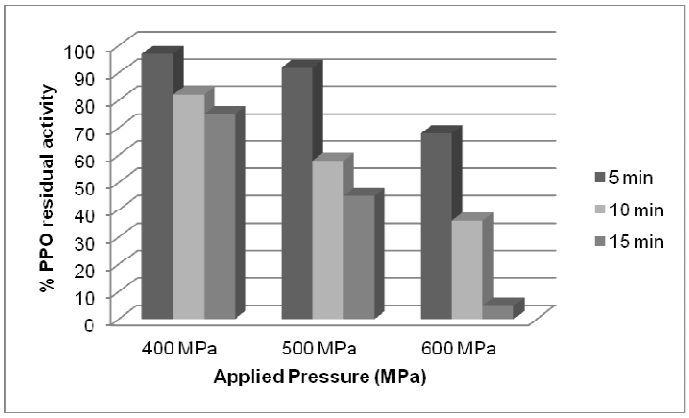

Fig. 2. Effect of high hydrostatic pressure treatments on PPO activity of avocado puree

Microbiological analysis

HHP treatment (600MPa, for 15 min at 20 ºC) of avocado-based salad prepared with a reduced pH (4.0) yielded microbiologically stable products during storage at 0, 5, 10 and 15 ºC. For all HHP treated samples stored at all temperatures, the measured colonies for total plate count and yeasts and moulds were below the detection limit, throughout the whole studied storage period. On the other hand, the untreated samples were spoiled within the first 5 days of storage at 5 ºC (total microflora higher than 10^7 CFU/g).

Colour change

Colour is considered to be the most deteriorative quality index for that kind of products. During storage, the colour of all samples was measured using appropriate equipment. The initial color parameters of the untreated (L=62.4, a=-7.02, b=35.18) and pressure-treated samples (L=61.59, a=-7.80, b=34.66) at 600 MPa for 15 min were not significantly different. During storage, only the a-value of the color parameters was altered significantly. The b value (yellow-blue parameter) and the L-parameter of the colour (higher L-value corresponds to brighter sample) of samples stored at 0, 5,10 and 15 ºC did not change significantly (Figure 3a). On the other hand, the a-value parameter (negative a-values represent green colour, while positive values represent red colour) appeared to be more sensitive to temperature and time. Storage at 15 ºC resulted in an increase of a-value from -8 to -1 within 7 days. At that time (when a-value equal to -1), the product was considered to be unacceptable. The effect of storage time and temperature on the a-values of all samples is depicted in Fig. 3b.

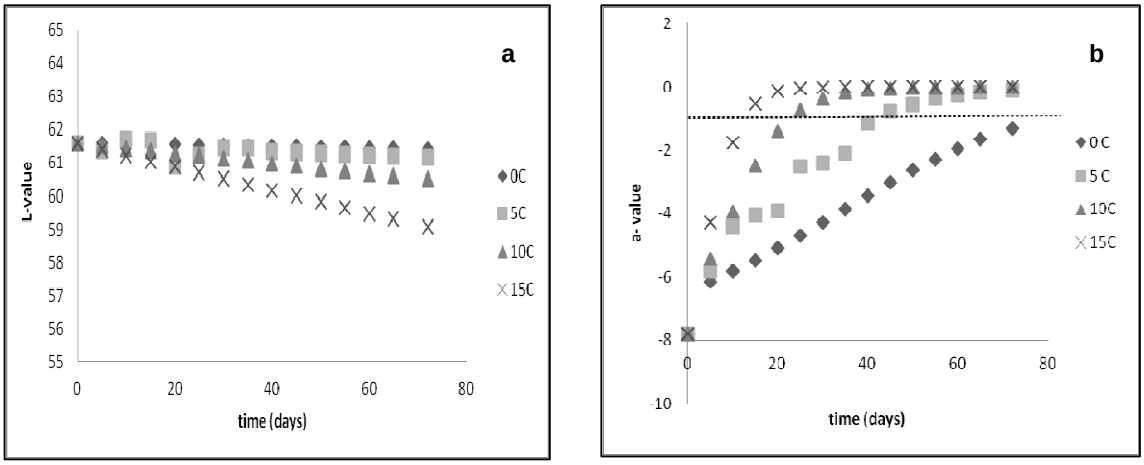

Figure 3. Colour parameters alteration: **a.** lightness (L-value) and **b.** red-green parameter (a-value) for the HP treated (600 MPa, 15 min, 20℃) product during storage at 0, 5, 10 and 15°C.

Sensory evaluation

All the samples were sensory evaluated by the sensory panel. According to their evaluation, HP-treatment did not significantly affect the overall impression of the samples compared to the untreated samples. For samples stored at 15 ºC, the degradation was fast, resulting in a non-acceptable product after storage of 7 days. When the samples were stored at lower temperatures the degradation was significantly slower. All the results of the samples scoring are depicted in Figure 4. The dot line represents the limit (score equal to 5) under which the samples are considered to be not appropriate for consumption. The limit was defined by the colour degradation of the samples (brown pigments appeared on the surface) and by the bitter taste that was developed to these samples.

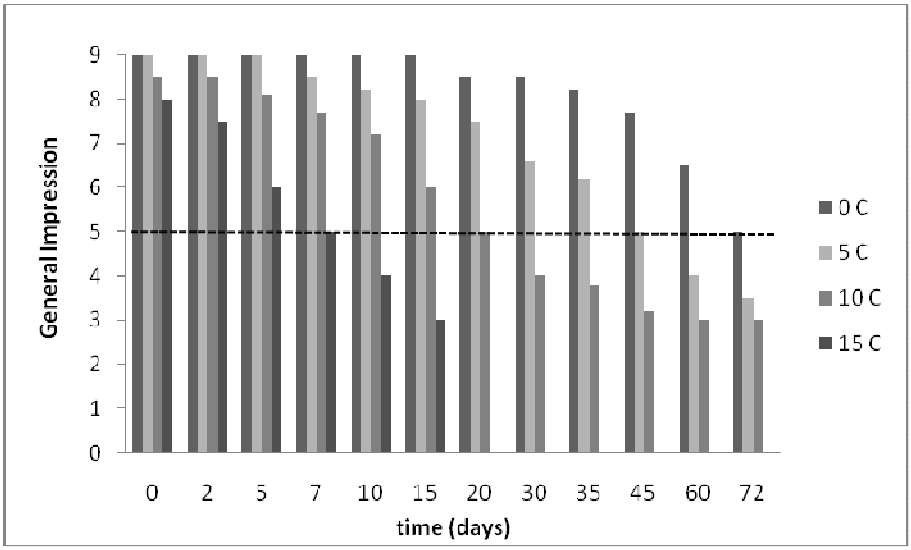

Figure 4. Sensory evaluation of high pressure-treated samples of "Green Mediterranean Deli Salad" during storage at 0,5,10 and 15 ºC

Shelf-life determination

The quality degradation and the shelf-life estimation of the developed product can be correlated to the development of bitterness in the product and by the colour alteration due to enzymatic browning. Taking into consideration all the received results from our study, the shelf-life can be determined based on colour alteration (a-value equal to -1) or the sensory evaluation (scoring equal to 5). The shelf life of this product is estimated as 72, 45, 20 and 7 days, after storage at 0, 5, 10 and 15 ºC, respectively. Comparing the obtained results with corresponding results for untreated samples stored at 5 ºC (shelf-life estimated as 5 days), a 9-fold increase is achieved when processing at 600 MPa at 20 ºC for 15 min.

CONCLUSIONS

The shelf-life study of a developed deli salad ready-to-eat product, HP treated at 600 MPA for 15 min, was studied. The HP treatment was used for the pasteurization of this product, in which mayonnaise emulsion was substituted by homogenized avocado puree which is very sensitive to enzymatic browning. The quality degradation and the shelf-life estimation of the developed product can be correlated to the development of bitterness in the product and by the colour alteration due to enzymatic browning. In general, the shelf-life was estimated as 72, 45, 20 and 7 days, after storage at 0, 5, 10 and 15 ºC, respectively.

The development of a commercially viable non mayonnaise based, vegetable deli salad with fresh-like quality and extended shelf life, named "Green Mediterranean Deli-salad", was achieved. The in pack non-thermal High Pressure (HP) processing, targeting on microorganisms and enzymes inactivation, while maintaining the sensorial quality of the food,

can be efficiently used. A superior product with commercial stability addressed to local and export markets was developed, based on the healthy image of avocado and Aloe Vera and other organically grown Mediterranean ingredients.

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PREDICTIVE MODELLING OF THE SHELF LIFE OF SMOKED FISH

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ABSTRACT: Smoking is a traditional method for preserving fish due to the synergistic action of salt incorporation, the preservative effect of smoke compounds and dehydration, creating a product with a characteristic flavour and an extended shelf life. The aim of the study was to model and comparatively assess changes in quality characteristics of smoked eel, mackerel and salmon, during refrigerated storage. Hot smoked eel (*Anguilla anguilla*) fillets, whole smoked mackerel (*Scomber japonicus*) and cold smoked salmon (*Salmo salar*) slices were provided by a Greek farming and processing unit. Eel fillets were stored isothermally at 0, 5, 10, 15℃ and at variable temperature conditions (T_{eff}=7.5℃) and mackerel and salmon slices were stored at 5 and 10℃. Quality assessment was based on microbiological analysis, colour, lipid oxidation (TBARs), TVB-N and sensory scoring. Quality indices were kinetically modelled and temperature dependence of quality loss rates was modelled by the Arrhenius equation. According to sensory evaluation, the shelf life was 7-9 weeks at 5℃ and 3-5 weeks at 10℃. The limiting TBARs value for sensory acceptability was 2 mg·kg^{-1} flesh for mackerel and salmon. Eel fillets exhibited higher TBARs values due to their significantly higher lipid content (23.9% crude lipids). TVB-N value of 10 mg N·100g^{-1} was used as the limit of acceptability for smoked eel. Results from variable temperature experiment were in good agreement with the prediction of the shelf life models developed from the isothermal experiments validating their applicability at the fluctuating storage conditions that usually occur in the cold chain.
Key words: *smoked fish, eel, mackerel, salmon, kinetic modelling*

INTRODUCTION

Smoking is one of the oldest means of preserving fish and meat. The long shelf life of smoked products is due to the low water activity, resulting from the added salt and slight dehydration, the processing temperature, which may be as high as 80-90℃ and the contribution of smoke components. In lightly smoked products, containing less salt and smoke components and more moisture, refrigeration and vacuum packaging contribute to preservation. Smoked seafood may contain up to 0.5 g/100 g of tissue of smoke components, mainly carboxylic acids and phenols, which have a distinct antimicrobial action. More susceptible to the action of smoke components are the vegetative forms of bacteria, while spores and moulds are usually more resistant.

Cold smoked fish is of considerable economic importance worldwide, particularly in Europe. These food products are produced by a light salting and smoking process (containing about 5.5% salt in the water phase and exposed at least 6-7 h to the action of smoke) and are typically consumed as ready-to-eat with no additional heat treatment. It is usually stored at chilled temperatures, is more sensitive than the hot smoked products and based on sensory evaluation have a limited shelf life of 2 to 6 weeks.

The process of smoking affects the sensory attributes of seafood by inducing desirable protein changes, resulting from the salt curing as in cold smoked products, or from heat treatment, e.g. in hot smoked eel. It imparts an appealing smoked colour and flavour to the fish. The colour of smoked fish depends mainly on the pigmentation of the skin, but is also affected by the amount and composition of the deposited smoke components and their interactions with the product. The kind of wood used for smoke generation has also a significant effect (Sikorski and Sun Pan, 1994).

European eel (*Anguilla anguilla*) is an economically important fish. The top three producing countries of farmed European eel are the Netherlands, Italy, and Denmark. Spain, Greece, Sweden and Germany are also centres of eel farming activity. The market demand for fresh eel has increased markedly due to its aroma and high yield. Eels are usually hot-smoked after brining or dry-salting, but the method differs considerably from country to country to suit local preferences. In Europe, eels are sold almost exclusively as skinned hot-smoked fillets.
Mackerel *(Scomber japonicus*) demonstrates an exceptional nutritional value in the human diet being rich in minerals, vitamins and polyunsaturated fatty acids. Mackerel is usually hot smoked to give a ready-to-eat delicatessen product. In Greece, mackerel is usually marketed as fresh chilled in ice or as processed, i.e. salted-smoked or canned. The smoked product can be whole gutted fish, with or without the head on, or fillets.
Salmon is a fish that is frequently consumed smoked (60% of total salmon produced). Vacuum packed cold smoked salmon is of considerable economic importance worldwide, and particularly in Europe. This product is produced by a light salting and smoking process and is typically consumed as ready-to-eat with no subsequent heat treatment. Thus, the salt concentration in the water phase of the product and proper refrigeration (below 5℃) along the whole chill chain are critical factors for product safety. The minimum salt concentration in the water phase of the product should be 3.5% (w/w), as measured in the thickest part of the fillet (FAO, 2005-2012).
The aim of the study was to model and comparatively assess changes in quality characteristics of hot smoked European eel (*Anguilla anguilla*) fillets, whole hot smoked mackerel *(Scomber japonicus*) and cold smoked salmon (*Salmo salar*) slices, during refrigerated storage.

MATERIAL AND METHODS

Hot smoked eel (*Anguilla anguilla*) fillets, whole smoked mackerel (*Scomber japonicus*) and cold smoked salmon (*Salmo salar*) slices were provided by a Greek farming and processing unit in their commercial (vacuum) packages. Samples were transported directly to the laboratory in polystyrene boxes with appropriate quantity of flaked ice (0℃) within 4–6 h. Eel fillets were stored isothermally at 0, 5, 10, 15℃ and mackerel and salmon slices were stored at 5 and 10℃ in high-precision (±0.2℃) low-temper ature incubators (Sanyo MIR 153; Sanyo Electric, Ora-Gun, Gunma, Japan), and their shelf lives were studied. Temperature in the incubators was constantly monitored with electronic, programmable, miniature dataloggers (Cox Tracer®, Belmont, NC, USA). Samples were taken in appropriate time intervals to allow for efficient kinetic analysis of quality deterioration.
In order to validate the applicability of the models from the isothermal experiments to real conditions, a variable temperature experiment was carried out on a separate batch of smoked eel fillets. A time-temperature scenario was applied, which consisted of several repeated cycles of three isothermal steps, 8 h at 3℃, 8 h at 10℃ and 8 h at 6℃. T_{eff}, the constant temperature that results in the same quality value as the variable temperature distribution over the same period, was calculated based on the Arrhenius model and the equivalent value was 7.5℃. Samples were taken at a ppropriate time intervals and the rates of the quality deterioration measured during the nonisothermal experiment were compared with the values calculated for T_{eff} by the models based on the isothermal experiments.
Quality assessment was based on microbiological analysis (total viable count, *Pseudomonas* spp., *Brochothrix thermosphacta*, *Lactobacilli*), colour, lipid oxidation (TBARs) and sensory scoring. Total aerobic viable count was enumerated on Plate Count Agar (PCA, Merck) after incubation at 25℃ for 72 h. *Pseudomonas* spp. were enumerated on Cetrimide Agar (CFC, Merck) after incubation at 25℃ for 48 h. *Brochothrix thermosphacta* was enumerated on STAA Agar (CM 881, Oxoid, Cambridge, UK) supplemented with SR 151 (Oxoid), which was incubated at 25℃ for 48 h. Lactic acid bacteria we re enumerated on De Man-Rogosa-Sharpe Agar (MRS, Merck) followed by incubation at 25℃ for 96 h. The microbial growth was modelled using the Baranyi growth model (Baranyi and Roberts 1995). For curve fitting, the in-house program DMfit of Institute of Food Research (IFR, Reading, UK) was used,

kindly provided by Dr J. Baranyi. Kinetic parameters such as the rate (k) of the microbial growth were estimated.

To evaluate lipid oxidation, 2-thiobarbituric acid reactive substances (TBARs) assay was performed according to the method of Loovas (1992). The absorbance was measured at 532 nm with a digital spectrophotometer (Unicam Helios; Spectronic Unicam EMEA, Cambridge, UK). The concentration of TBARs as calculated from a standard curve prepared by 1,1,3,3-tetraethoxypropane and expressed as mg malonaldehyde (MDA) per kg.

Total volatile basic nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N) analyses were conducted on a single TCA extraction by distillation in a Kjeldahl rapid distillation unit (Buchi 321 Distillation unit, Flawwil, Switzerland) and titration with sulphuric acid (Pivarnik et al. 2001). For the determination of TMA-N, 20 ml formaldehyde 37% was added in the distillation tube to block the primary and secondary amines.

The sensory parameters (appearance, odour, texture, taste) of smoked fish were evaluated by a trained sensory panel of eight and sensory scores were recorded in appropriate forms, reflecting the organoleptic evolution of quality deterioration. Additionally, panellists were asked to score the overall impression and acceptability. Rating was assigned separately for each parameter on a 1–9 descriptive hedonic scale (9 = like extremely and 1 = dislike extremely). A sensory score of 5 was taken as the average score for minimum acceptability.

Values of the different measured indices were plotted against time for all temperatures studied, and the apparent order of quality loss was determined based on the least-square fit. Temperature dependence of the deterioration rate constants, k, was modelled by the Arrhenius equation (Eq. 1)

$$\ln(k) = \ln(k_{ref}) - \left(\frac{E_a}{R}\right)\left[\frac{1}{T} - \frac{1}{T_{ref}}\right] \quad (1)$$

where k_{ref} is the rate constant of the degradation of the respective quality index at a reference temperature, T_{ref} (e.g. 4℃ for refrigerated foods), T is the temperature in K, E_a is the activation energy of the studied action and R is the universal gas constant.

RESULTS AND DISCUSSION

Table 1 shows the composition (nutrition information) of hot smoked eel, hot smoked mackerel and cold smoked salmon. These fish products are excellent sources of high biological value protein and omega-3 fatty acids (ω3-FA).

Table 1. Nutrition information per 100g of fish product

	Hot smoked eel	Hot smoked mackerel	Cold smoked salmon
Energy	1258kJ/301kcal	668kJ/159kcal	885kJ/212kcal
Protein	21.4 g	28.0 g	27.9 g
Fat	23.9 g	5.3 g	11.1 g
SFA	6.4 g	1.6 g	1.7 g
ω3 fatty acids	4.1 g	1.8 g	1.9 g
20:5 ω3 EPA	1441 mg	294 mg	379 mg
22:6 ω3 DHA	1404 mg	1215 mg	556 mg
Na	0.9 g	0.7 g	0.3 g

Results are given as mean ± standard deviation (n = 3); SFA - saturated fatty acid; EPA - eicosapentaenoic acid; DHA - Docosahexaenoic acid

Hot smoked eel

Total plate count and lactic acid bacteria counts showed a slow increase during refrigerated storage reaching a plateau at 4 logcfu/g after 50 days at 0 and 30 days at 5℃. *Pseudomonas* spp. and *Brochothrix thermosphacta* were not detected (<100 cfu/g) at all storage temperatures. Sensory scorings and TBA values were modelled with apparent zero order equations. TVBN values increased with storage time and were modelled with apparent first order equations. The E_a values for the rates of TVBN, total plate count, lactic acid bacteria growth and TBA increase were 136.3, 51.0, 81.2 and 74.4 kJ/mol, respectively

(Figure 1a). Overall impression scores showed high correlation with TBA and TVB-N values. At all temperatures studied, the time of sensory rejection coincided with TBA concentration of 15 mg MDA/kg. TVB-N level of 10 mg·100g^{-1} flesh could be regarded as the limit of acceptability for smoked eel. This is in agreement with Özogul et al. (2005) who reported the limit of 10 mg N·100g^{-1} for European eel stored in ice. The shelf life of smoked European eel determined by sensory evaluation was 82, 54, 20 and 12 days at 0, 5, 10 and 15℃, respectively.

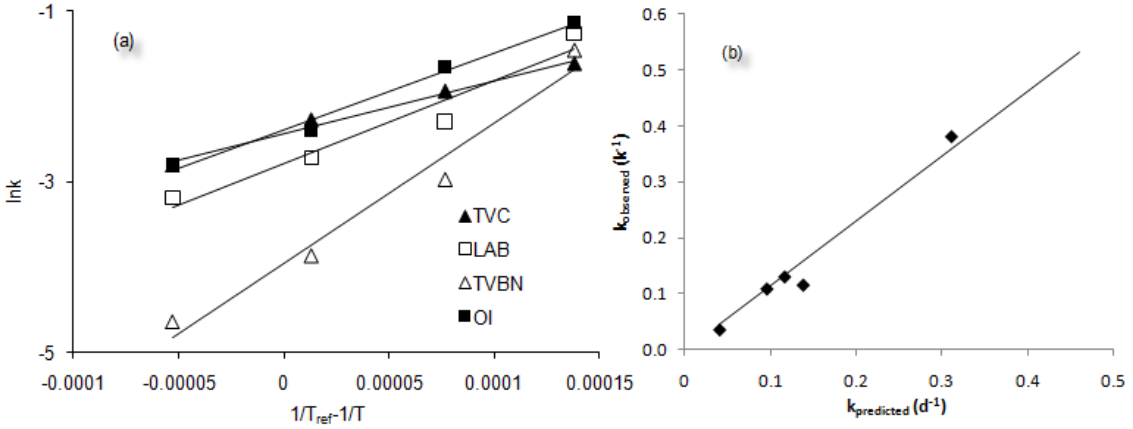

Figure 1. (a) Arrhenius plots of the rates of quality deterioration (TVC - total viable count, LAB – lactic acid bacteria, TVBN – total volatile basic nitrogen, OI – overall impression (sensory scoring), (b) Rates of chemical and sensory changes determined by the non-isothermal experiment ($k_{observed}$) and calculated by the models from the isothermal experiments ($k_{predicted}$)

Results from variable temperature experiments were in good agreement with the prediction of the shelf life models developed from the isothermal experiments, validating their acceptability at the fluctuating storage conditions that usually occur in the cold chain (Figure 1b). Relative errors averaged at ±13% (the highest value was -19.2%) for all the indices measures, which is below the 20% limit used in the literature as criterion of acceptability (Gougouli et al., 2008).

Hot smoked mackerel and cold smoked salmon

Slow microbial growth was observed at 10℃, with total viable count reaching a value of 5 logcfu/g after 50 days for both fish products. Sensory scorings for mackerel and salmon were modelled with apparent zero order equations. According to sensory evaluation, the shelf life was 8-9 weeks at 5℃ and 3-5 weeks at 10 ℃. TVB-N values showed small changes and remained in low levels for mackerel and salmon stored isothermally at 5 and 10℃ (<10 mg·100g^{-1} sample). Organoleptic degradation was well correlated with lipid oxidation. The limit TBARs value for sensory acceptability was 2 mg·kg^{-1} flesh for mackerel and salmon. This value is lower than the respective TBARs values reported by Nishimoto et al (1985) for high and low quality mackerel (i.e. 4 and 27 mg MDA·kg^{-1}, respectively).

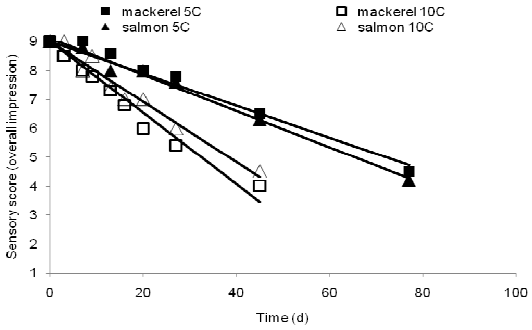

Table 2. Shelf life (in days) of hot smoked mackerel and cold smoked salmon stored isothermally at 5 and 10℃, calculated based on TBARs value[1]

	Hot smoked mackerel	Cold smoked salmon
5℃	72	64
10℃	32	38

CONCLUSIONS

The objective of the study was to model and comparatively assess changes in quality of hot smoked European eel and mackerel and cold smoked salmon, during refrigerated storage. According to sensory evaluation, the shelf life was 7-9 weeks at 5℃ and 3-5 weeks at 10℃. Eel fillets exhibited higher TBARs values due to their significantly higher lipid content (i.e. 24% crude lipids compared to 5.3 and 11.1% for mackerel and salmon, respectively). The results from the non-isothermal experiment showed a satisfactory agreement with the prediction based on the kinetic models developed from the isothermal experiment and the rates of change of the quality indices are not dependent on the temperature history. Of course the starting value of any indice will affect the time to reach the upper acceptable level, i.e. shelf life, and this parameter is taken into account in the developed models.
If the temperature conditions of the products could be continuously monitored, e.g. by inexpensive Time-Temperature Integrators, reliable estimation of the quality status and the remaining shelf life of the products could be performed based on the presented modelling of the quality indices. This could allow better management and optimization of the cold chain from manufacture to consumption (Taoukis et al., 2012).

ACKNOWLEDGEMENTS

Special thanks to V. GEITONAS & Co Ltd (Arta, Epirus, Greece) for financing and providing the fish products.

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EFFECT OF CONTAINERS AND PACKAGING ON THE QUALITY OF GRAIN MILLING AND BAKERY PRODUCTS

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ABSTRACT: Alterations in quality of food during storage in most cases have negative effects on sensory and biological values of products.
Packaging must maintain the quality of a product unchanged from the moment of packing until consumption. Barrier properties of packaging materials are very important for this purpose. These properties must protect the products from the effects of the environment such as air, especially oxygen, which may cause oxidation of some product components when exposed to light.
Depending on the moisture content, bakery products show various signs of aging. Products with higher initial content of moisture, with soft crumb when fresh, lose elasticity and harden during aging with undesirable changes of odor and taste. Flour products with low initial content of moisture absorb the ambient moisture during aging; they soften, become sinewy and stale. The problem is also microbiological contamination of bakery products, particularly molds which are most often causes of deterioration of this group of products.
The paper gives a review of the research results on grain milling and bakery products shelf-life. Wheat flour T-500, wheat semolina, toast bread and croissants were tested, i.e. shelf-life of these products was controlled in their standard packaging. The obtained results indicate that packaging used in this research did not affect the shelf-life of the tested products. They also showed that the right selection of packaging and determination of optimal procedure of packaging and bagging of grain milling and bakery products assured optimal period of shelf-life of these products and did not shorten it.
Key word: *containers, packaging, wheat flour, wheat semolina, toast bread, croissants.*

INTRODUCTION

Bakery products are very sensitive organic origin substrate of organic origin which undergo physical and chemical processes causing the change in quality and sensory, chemical and nutritional values during the period of storage (Gvozdenović, 2010).
During storage certain alterations in texture of bakery products or their taste may occur. Bread becomes dry and hard due to starch retrogradation. Variety of grain milling and bakery products as well as their complex composition are both a specific problem of their preservation. Both their appropriate production and preservation of product quality are important. To achieve this it is necessary to wrap and pack the products in appropriate wrappings and packaging which will protect the products from scattering and changes caused by external environmental effects.
To reduce the undesirable changes in quality of these products to the lowest possible level and thus provide good quality for appropriate storage period, it is necessary to know qualitative properties of material and wrapping along with qualitative properties of the wrapped content.
The packaging must maintain the quality of the product unchanged from the moment of packing until the moment of consumption. Barrier properties are very important for this purpose since they must protect the product from effects of environment, such as air and especially oxygen, which may cause oxidation of some product components when exposed to light (Gvozdenović et al.,1999).

The purpose of this paper is to test effects of certain types of grain milling and bakery products packaging on alterations in physical, chemical and sensory properties of tested samples.
Packaging material used for packaging of fresh bread, sliced bread and pastry with extended shelf-life should be with as little water vapor permeability as possible in order to prevent drying. Besides, the occurrence of moisture condensation should be considered. Apparently, vapor condensation on the inner side of the foil has a direct effect on quality alteration due to temperature reduction – it causes crust softening and favors mold growth.
Oxidation, specific aroma loss and stale odor and taste may occur with products having higher fat content (Crnčević,1980).

MATERIAL AND METHODS

The bakery products, being wheat flour T-500, wheat semolina, toast bread and croissants, produced in A.D. Žitoprodukt Zrenjanin were tested.
Physical and chemical analysis and sensory assessment of grain milling and bakery products were performed in accordance with Regulation on methods of physical and chemical analysis for quality control of wheat, grain milling and bakery products 1988, their freshness i.e. the shelf-life of these products in their standard packaging was tested. Unlike bakery products whose quality was controlled for several days (in accordance with the product label), the quality of grain milling products was controlled during a one-year period of time.
Wheat flour T-500 was sacked in two-layer 25 kg kraft paper sacks sewed up, wheat semolina T-400 in one-layer 1 kg kraft paper bags, toast bread and croissants were wrapped in polypropylene foil. Five sacks of flour and three samples from each wheat semolina, toast bread and croissants were analyzed.

RESULTS AND DISCUSSION

The results of physical and chemical and sensory assessment of wheat flour T-500 are shown in table 1 and figure 1.

Table 1. Shelf-life testing of wheat flour T – 500

Date	25/01/2010	22/04/2010	20/07/2010	20/10/2010	20/01/2011
Moisture content %	14.85	14.38	14.07	13.82	13.62
Gluten content % in dry substance	25.5	25.1	24.9	24.1	23.2
Sensory assessment	5	5	4	4	3

The obtained results show that in sacked wheat flour T – 500 the moisture content was slowly reduced. The change in moisture of flour depends on both the protection properties of packaging material and relative moisture and air tepmerature in the storehouse. When flour is stored in sacks arranged in rows, mositure will change at a slower rate.
The content of moist gluten was also reduced, which indicates that there are changes of certain components of protein-proteinase complex of flour during storage (physical properties of gluten change leading to reduction of flexibility) (Đaković, 1997). Reduction of gluten content results in lower technological quality of flour.
As for the sensory assessment, the characteristic flour odour weakened and small lumps in the last analysed sample were visually detected.
In wheat semolina, Table 2, the moisture content was lower, acid level was higher between the second and the third test.

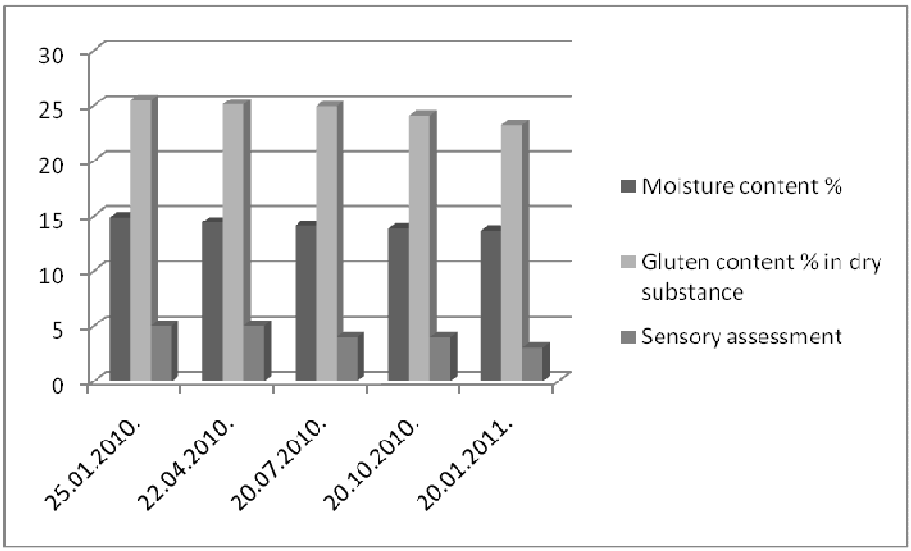

Figure 1. Diagram of wheat flour T – 500 shelf-life

Acid level increases due to amassing of free unsaturated fatty acids (Auerman, 1988). According to the Regulation on quality of wheat, grain milling and bakery products, 2004, the level of acide with wheat semolina T-400 is considered regular if lower than 2.5.
A brighter shade of the last analysed sample was noticed. The color of flour comes from pigment and depends on the color of grain endosperm and quantity of peripheral parts of the grain (Kaluđerski et al.,1998). Since the tested samples were packed in bags, wheat semolina bleaching is slow and practically may be noticed only when stored for a longer period of time.

Table 2. Shelf-life testing of wheat semolina T-400

Date	20/07/2010	20/10/2010	20/01/2011
Moisture content %	14.98	13.98	13.19
Acid level	1	1.11	1.44
Sensory assessment	5	5	4

The results presented in Table 3 containing data about physical and chemical analysis and sensory assessment of toast bread show that there were aroma loss and changes in texture of bread. Decrease in total content of water due to water diffusion from the inner part to external crust was also noticed.
The odor of the first analyzed sample is very intense, pleasant, characteristic for this type of bread as well as the taste which is intense, solubility of crust and crumb is excellent, the crumb is sticky and does not crumb.

Table3. Shelf-life testing of toast bread

Date	20/01/2011	22/01/2011	24/01/2011
Moisture content of toast bread crumb %	41.96	40.21	39.34
Acid level of crumb	1.56	1.56	1.58
Sensory assessment	5	4	3

The change in quality of croissants, Table 4, is a result of freshness loss. The increased content of fat may cause changes in quality due to fat oxidation during longer storage period. Temperature, presence of air i.e. oxygen, light and lipolytic enzymes have effects on oxidative stability of the product (Gvozdenović et al., 2010).

Table 4. Shelf-life testing of croissants

Date	20/01/2011	22/01/2011	24/01/2011
Moisture content of croissants %	23.80	23.20	22.90
Fat content % in dry substance	20.70	21.00	21.40
Sensory assessment	5	4	2

CONCLUSION

The results of the performed tests lead to the following conclusions:

- analyses of flour quality indicate the necessity to undertake laboratory analyses of raw material and consideration of flour quality prior to its use in bakery production
- since the grain milling products are very sensitive to changes of moisture and gluten content, the selection of packaging material is of utmost importance
- due to sensitivity to moisture, bakery products need wrapping which could provide optimal protection of wrapped product from mechanical effects and changes of nutritional quality of the product
- in order to obtain more reliable results of the quality of bakery products and to provide their shelf-life it is necessary to select adequate types and combinations of packaging materials and undertake control of all parameters of technological process
- the right selection of packaging material and determination of optimal packaging and bagging procedure of grain milling and bakery products also provide optimal shelf-life period of those products and does not shorten it.

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EFFECT OF PACKAGING METHOD AND STORAGE TIME ON LIPID PEROXIDATION AND FATTY ACID COMPOSITION OF SERBIAN TRADITIONAL *PETROVSKÀ KLOBÀSA* SAUSAGE

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ABSTRACT: The influence of two packaging methods, under vacuum and modified atmosphere packaging, (MAP) and of storage time (45 and 270 days of production) on lipolytic and oxidative reactions and on fatty acid composition of the serbian traditional *Petrovská klobása* sausage were compared. Sausages were household produced and subjected to smoking, drying and ripening processes for 45 days. They were then divided in three experimental groups. The first group consisted of unpackaged sausages, while the second and third groups consisted of sausages packaged under vacuum and in MAP (80% N_2 and 20% $C0_2$), respectively. Intensity of lipolysis in sausages was estimated by the value of acid number (KOH/g lipids) and oxidative changes were monitored through the concentration of malondialdehyde (MDA). These parameters were examined at the end of the drying process (45^{th} day of production) and at the end of the storage period (270^{th} day of production). Acid number at the end of drying process was significantly ($P<0.05$) lower, while MDA content and fatty acid composition of total lipids did not differ significantly compared to that of unpackaged sausages after 270 d storage. At the end of the storage time, acid number for unpackaged sausages (21.83 mg KOH/g lipids) was significantly ($P<0.05$) higher than for sausages packaged under vacuum (15.03 mg KOH/g lipids) and in MAP (16.90 mg KOH/g lipids). Based on MDA content at the end of the storage period, lipid peroxidation intensity was significantly ($P<0.05$) higher in unpackaged sausages (0.85 mg MDA/kg fresh sausage), whereas fatty acid composition did not differ significantly comparing to that of sausages packaged under vacuum (0.38 mg MDA/kg) or in MAP (0.53 mg MDA/kg). Thus, it can be concluded that traditional *Petrovská klobása* sausage can be successfully stored using vacuum or MAP packaging without any significant alterations in terms of sensitivity of their lipids to lipolysis and of their fatty acids to peroxidation.
Key words: *traditional sausage, packaging, vacuum, modified atmosphere, lipolysis, peroxidation, fatty acids*

INTRODUCTION

Nowadays in many european countries, the demand for traditional food products has increased. Moreover, food and gastronomy form an inherent link with tourism in Europe, with a renewed interest of consumers for typical and regional foods (Talon et al., 2007). Traditional fermented food products of meat origin are produced by numerous small-scale and family sized processing units in Europe. Their fermentation relies on natural contamination by environmental flora. This contamination occurs during animal slaughtering and increases during manufacturing. Fermentation is not controlled (Tasić et al., 2012). Petrovská Klobása (Petrovac Sausage) is a traditional dry fermented sausage from Bački Petrovac (province Vojvodina, Serbia), which has been gained much popularity in last years in Serbia and in near by countries, even further. Due to its specific and recognizable sensorial qualities (texture, flavour and taste), this product is protected with the mark of geographic origin under Serbian law (Petrović et al., 2007). It is produced exclusively from pork meat and fat, red hot paprika powder, salt, crushed garlic, caraway and sugar leading to specific hot taste, aromatic and spicy flavors, a dark red color and a firm consistency (Ikonić et al., 2010). Dry fermented sausages are meat products with a high fat content (20-40%).

Fat influences the nutritional and health values of dry fermented sausages. It acts as a source of essential polyunsaturated fatty acids (PUFA) and fat soluble vitamins and constitutes a very concentrated source of energy in the human diet (9 kcal/g) (Olivares et al., 2010). However, fermented sausages show some negative aspects as a consequence of their high animal fat content. Namely, events that altered fat structural stability during processing fermented sausages, such as lipolysis and lipid oxidation, have a major impact, both desirable and deleterious for the final product quality of meat products. Lipid oxidation can altered sensorial properties of food products, since fat contributes to flavour, texture, mouth feel, juiciness and overall sensation of lubricity of the product (Muguerza et al., 2002). Besides, oxidation can affect the nutritional value of food by decomposition of vitamins, oxidation of essential PUFA leading to production of peroxides (malondialdehyde, hydroxy-alkenals,..) very toxic compounds for consumers (Ansorena and Astiasarán., 2004).
Retail storage of dry fermented sausages is usually done in aerobic conditions, the product being exposed to oxygen and, in consequence, to a potential risk of lipid oxidation. Furthermore, an excessive dehydration can also ocur using traditional storage conditions, leading to economic loss because of weight loss. Consequently, industrial trends are to extend the shelf life of this type of product by adopting improved storage practices (Ansorena and Astiasarán., 2004). Both modified-atmosphere and vacuum packaging are a commercial way for the retail selling of dry fermented sausages. Valencia et al., 2006 and Rubio et al., 2008 have reported the effect of the packaging conditions (vacuum and modified atmosphere) of dry fermented sausages on oxidation of their lipids.
Taking into account all negative effects of drying and storage on lipid structure of fermented sausages , our present study was designed to determine the effect of different conditions of packaging and storage time on theintensity of lipolytic and oxidative reactions occuring in traditional *Petrovská klobása* sausages produced in industrial conditions.

MATERIAL AND METHODS

Petrovská klobása sausages were prepared according to the procedure described by Tasić et al. (2012). They were elaborated in industrial conditions of smoking (a sequence of 12 treatments of 30 min each) during 3 days followed by a drying (temperature: 10^0C; relative humidity: 75-90%) for 45 days (% moisture: < 35%), Sausages were then divided in three experimental groups. The first group consisted of unpackaged sausages, whereas sausages of the second and third groups were packaged under vacuum and in MAP (80% N_2 and 20% $C0_2$), respectively. After packaging, sausages were stored in a chamber with a controlled temperature (15℃) and relative humidity (75%) for 225 days. Analyses of fatty acid composition of total lipids and of lipolytic level as well of oxidative level were conducted after 45 days of drying and 225 days of storage. All determinations were made in duplicate for three samples of each batch. Means were compared by Duncan's multiple range test, using the STATISTIKA 8.0 computer programme (2008).
Fatty acids were determined from total lipids extracted from sausages. The Folch et al. (1957) method was used for lipid extraction. Boron trifluoride/methanol was used for the preparation of fatty acid methyl esters (FAME) (Bannon et al., 1982) for the fatty acid composition of total lipids determined by gas chromatography with the Perkin–Elmer Autosystem XL gas chromatograph fitted with a capillary column Omegawax 320 and flame ionization detection. The injector and detector temperatures were both of 260^0C. The carrier gas was helium, at the pressure of 11 psi. The sample volume was 0.5µl. FAME was identified by their retention time in comparison with those of FAME standards separated in same chromatographic conditions. Centesimal distribution of fatty acids was calculated as a weight percentage of total FAME.
Acid number of total lipids (mg KOH/g lipids) was quantified according *SRPS ISO* (2000). The TBARs test was used for determination content of malonaldehyde (MDA) (Botsoglou et al., 1994).

RESULTS AND DISCUSSION

In this work, the fatty acid profile did not show significant changes after storage in all conditions tested (Table 1). After 270 days of production, no significant differences in lauric (C12:0), stearic (C14:0), palmitoleic (C16:1) and oleic (C18:1) acids were observed among batches (P>0.05). All sausages of three batches maintained lower values comparing to sausages at the end of the drying process after 45 days. Therefore, content of linoleic (C18:2 n-6) and linolenic (C18:3 n-3) acids ranged between 13.94-14.15% and 0.32-0.37%, respectively, these values being not significantly changed even after 225 days of storage (P>0.05). Level of total saturated fatty acids (SFA) was stable and similar in all batches, ranging between 35.27-35.96%. Also, no significant differences were found among batches for total unsaturated (UFA) and polyunsaturated fatty acids (PUFA) (P>0.05) (Table 1).

Table 1. Effects of packaging treatment and of storage time on fatty acid composition in total lipids of serbian traditional *Petrovská klobása* sausage.

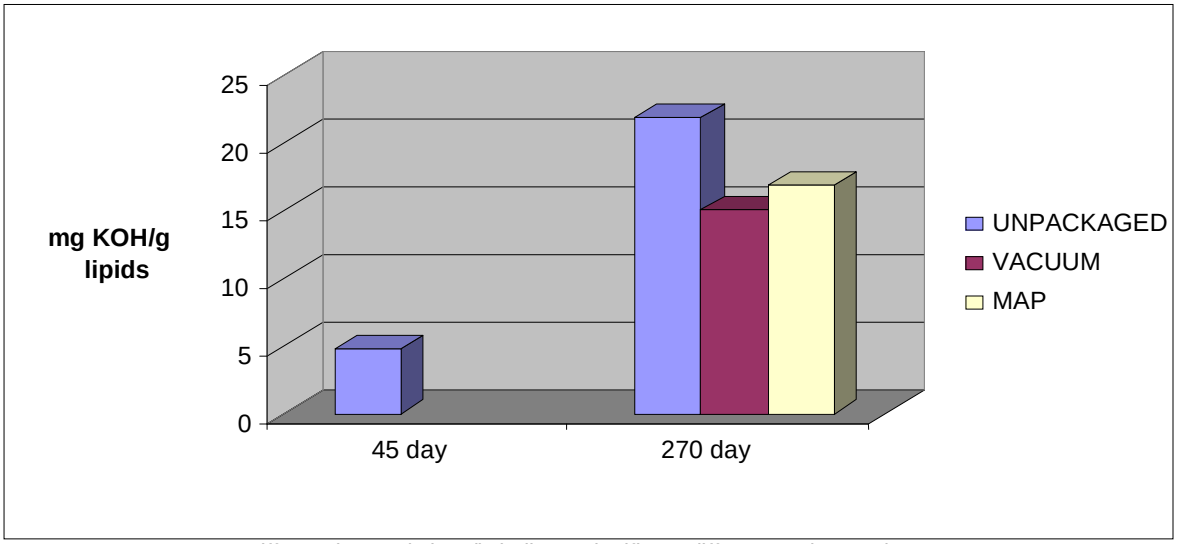
Fatty acids	End of drying	End of storage time		
		Unpackaged	Vacuum	MAP
C12:0	0.27 ± 0.03	0.30 ± 0.02	0.31 ± 0.01	0.33 ± 0.01
C14:0	1.40 ± 0.04	1.42 ± 0.04	1.55 ± 0.10	1.52 ± 0.02
C16:0	20.22 ± 0.10	20.51 ± 0.30	20.46 ± 0.19	20.50 ± 0.22
C18:0	13.05 ± 0.23	13.32 ± 0.29	12.96 ± 0.02	13.29 ± 0.01
C20:0	0.33 ± 0.03	0.32 ± 0.02	0.33 ± 0.02	0.32 ± 0.04
C14:1	0.19 ± 0.04	0.22 ± 0.02	0.26 ± 0.01	0.25 ± 0.02
C16:1	4.92 ± 0.10	4.77 ± 0.13	4.51 ± 0.12	4.85 ± 0.12
C18:1	41.57 ± 0.30	40.98 ± 0.39	40.80 ± 0.14	40.78 ± 0.12
C20:1	0.90 ± 0.05	0.84 ± 0.02	1.04 ± 0.04	1.08 ± 0.01
C18:2	13.94 ± 0.10	14.11 ± 0.14	14.02 ± 0.01	14.15 ± 0.15
C20:2	0.22 ± 0.06	0.29 ± 0.03	0.26 ± 0.02	0.28 ± 0.04
C18:3	0.35 ± 0.03	0.32 ± 0.01	0.37 ± 0.03	0.36 ± 0.01
C20:4	0.22 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.22 ± 0.01
∑SFA	35.27 ± 0.43	35.87 ± 0.04	35.61 ± 0.01	35.96 ± 0.08
∑UFA	62.51 ± 0.02	61.77 ± 0.01	61.51 ± 0.02	61.97 ± 0.06
PUFA	14.93 ± 0.13	14.96 ± 0.06	14.90 ± 0.04	15.01 ± 0.04
UFA/SFA	1.72 ± 0.02	1.72 ± 0.00	1.73 ± 0.00	1.73 ± 0.00

SFA- saturated fatty acids; UFA- unsaturated fatty acids; PUFA-polyunsaturated fatty acids. Results are expressed as mean ± standard deviations. Values in the same row bearing different letters are significantly different (P< 0.05).

These results pointed out the high stability of fatty acid distribution in total lipids of sausages in these storage conditions. Similar results were already reported by Rubio et al., 2008 and Valencia et al. 2006. Also, in dry fermented sausages, Valencia et al. (2007) did not find any differences for FA distribution at the end of drying and those obtained after 30 and 90 days of storage under vacuum.

Lipids are associated to many aromatic substances generated by lipolytic and oxidative phenomena taking place during the ripening process (Navarro et al., 1997). The intensity of lipolytic and oxidative phenomena in the studied batches was determined by parameters related to lipid stability such as acid number (Graph 1). Lipolysis of sausage lipids led to the increase of free fatty acid content attributed at the beginning of fermentation to both microbial and muscle lipases acting in the sausages (Visessanguan et al., 2006).

The acid number of the lipids in Petrovska klobasa sausages ranged from 4.79 to 16.90 mg KOH/g of lipids (Figure 1) in accordance with literature data (Vuković et al., 2011). At the end of drying process, the acid number in sausages (unpacked sausage - 45 day) had significantly (P<0.05) lower values than those of all batches at the end of storage. Similar results for this type of sausage were reported (Sumo et al., 2010, Visessanguan et al., 2006).

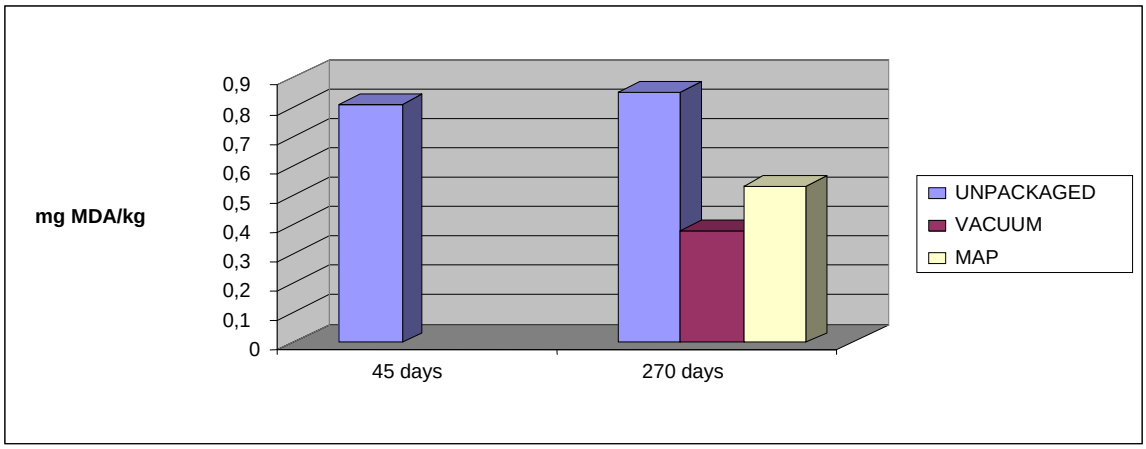


Different letters (a,b,c,d), indicate significant differences (P<0.05).

Figure 1. The effect of packaging treatment and storage time on of acid number values (mg KOH/g lipids) in serbian traditional *Petrovská klobása* sausage

Additionaly, differences in the acid number values were found between packaging methods (P<0.05). Namely, vacuum packed sausage had significant lower acid number (15.03 mg KOH/g lipids) comparing to those of unpackaged (21.83 mg KOH/g lipids) and MAP (16.90 mg KOH/g lipids) packaged sausages. These results showed the lowest hydrolytic changes in vacuum packaged sausage, but this observation was in contrast with a literature data (Visessanguan et al., 2006).

Malonaldehyde (MDA), the main degradation product of lipoperoxidation, was used as the marker for lipid peroxidation intensity. In contrast to acid number values, TBA values decreased during storage in accordance with literature data. Namely, Janero et al., (1990) pointed out that a decrease in TBA values during storage could be attributed to MDA reaction with amino acids, sugars and nitrite in complex formulations. The results of TBARS test, expressed as MDA content (mg/kg), are presented in Figure 2. Our TBA values were in accordance with those of Ansorena and Astiasarán (2004), but much lower than those of others for the same type of product reported by Nassu et al., (2003) and Rubio et al.(2008). At the end of drying process, (45th day), values of MDA in unpackaged sausage amounted to 0.81 mg/kg fresh product to values not significantly different (P>0.05) compared to those of unpackaged sausage (270days) (0.85 mg/kg), but significantly higher than those in vacuum (0.38mg/kg) and MAP (0.53mg/kg) packaged sausages at the end of storage.



Different letters (a,b,c,), indicate significant differences (P<0.05).

Figure 2. The effect of packaging and storage times on TBARs values (mg/kg) in serbian traditional *Petrovská klobása* sausage

Considering storage conditions, differences were found in the TBA values between packaging methods. Namely, batch packaged under vacuum shown lower TBA values (P <0.05) than those found in gas packaged samples. Similar results for this type of sausages were already reported by Zanardi et al. (2002).

CONCLUSIONS

Sausages packaged under vacuum or MAP indicated a significant lower (P<0.05) of lipolytic and oxidative changes during storage, comparing to that with unpackaged sausages. Therefore, it can be concluded that the *Petrovská klobása* sausage can be stored for a long period using either vacuum or modified atmosphere packaging without any defavorable effects on the nutritional and health values properties.

ACKNOWLEDGEMENTS

The research programme was financed by the Ministry of Education and Science of the Republic of Serbia, Project No. TR 31032.

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STUDY ON EFFECTS OF STORAGE CONDITIONS AND PET PACKAGING ON QUALITY OF EDIBLE OILS

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ABSTRACT: The Effects of polyethylene terephthalate (PET) pieces and different storage conditions on fatty acids profile and some quality factors in three types of commercial oils, namely sunflower oil, canola oil, and blended oil containing sunflower oil, soy bean oil, and cottonseed oil were studied. Quality factors such as peroxide value, free fatty acids, and iodine value and induction period before and after storage of samples at 25 and 45℃ for 20 and 60 days in the presence or absence of PET pieces were investigated. It was concluded that the stability of vegetable oils is dependent on the type of oil and its initial physical and chemical properties, time and temperature of storage and the type of employed packaging material (PET and glass). This study showed that storing oils at low temperatures (T<25 ℃) may be recommended in order to extend the shelf life and maintenance quality of commercial oils which are packaged in PET containers.
Key words: *canola oil, fatty acid profile, blended oil, PET pieces, sunflower oil, quality factors*

INTRODUCTION

Oils play a crucial role in the human diet. More than 90% of the world oil production from vegetable, animal and marine sources is used in foods or as part of it (Tawfik and Huyghebaret, 1999). Different kinds of oil have various uses in the food industry, among them canola and sunflower oils are used extensively for cooking. The blended oil (which contains sunflower oil, soy bean oil, and cottonseed oil) is considered as one of the current oils in Iran's market. The materials which are used for packaging have a large variety. Glass, metal and different kinds of plastics are used in oil packaging (Tsimis and Karakasides, 2002; Tawfik and Huyghebaret, 1999). The kinds of packaging have significant affect on shelf life of the oils as the carefully processed oil may be damaged by inelegant selection of packaging materials (Ramazani, 2007). PET is one of the most commonly used plastics in food packaging covering a wide range of packaging material. PET satisfies many important requirements such as good aesthetic aspect (brilliance and transparency); suitability for coloring; good mechanical, thermal, and chemical resistance; low production cost; suitability for short storage, easy recyclability, and low weight with respect to glass bottles (Tsimis and Karakasides, 2002). The trend toward incorporating modifier compounds into PET packaging resins has grown in order to produce containers with a high degree of clarity, in a wide variety of custom shapes, and free from residual acetaldehyde. In addition, incorporation of antioxidant stabilizers in PET increases its application in the food area, particularly for vegetable oil storage (Piergiovanni and Limbo, 2010; Tsimis and Karakasides, 2002). Important characteristics such as barrier properties of packaging materials against moisture, oxygen and the interaction between foodstuff and packaging materials have an important effect on the quality and shelf life of oils in foodstuff (Sharma *et al*., 1990). Hence, the major function of packaging is to minimize the reaction which has direct effect on stability of the contained product. The studies published about the effect of packaging on oil quality have concluded that stability can be enhanced by suitable selection of packaging material (Leo,

1983; Kaya *et al*., 1993; Kucuk and Caner, 2005; Ramazani, 2007; Coutelieris and Kanavouras, 2005; Tsimis and Karakasides, 2002; Tawfik, 2005).
One of the additives, fat soluble phenolic antioxidants, present in the compounded polymer may migrate at an appreciable rate into the oil during storage which then effect its stability (Cinquanta *et al*., 2001). Numerous studies have shown the release of antioxidants from the packaging materials into oil-containing foodstuff (Baner *et al*., 1992; Niebergall *et al*., 1993). The extension of shelf life of some products due to the presence of such compounds has been reported (Miltz *et al*., 1988). The shelf life and oxidative stability of olive oil stored in glass and polyethylene (PE) bottles were examined (Kiritsakis, 1984). Due to this research, glass bottles which prevent oxygen from entering the oil were more protective against oxidation in comparison with plastic bottles (PE). Kaya *et al.* (1993) studied the effect of permeability and transparency of the packaging materials (glass and PET bottles) on the shelf life and also stability of sunflower and olive oils. In this research, oxidative stability of the oil was studied by measuring the peroxide values. The results demonstrated that storage stability of oil increased in the order which depended on packaging material, colored glass > transparent glass > PET..Satue *et al.* (1995) reported that the extent of oil oxidation is often specified by measuring the peroxide value (PV). Since this index is related to the hydroperoxides, the primary oxidation products which cause rancid flavor because of their instability and hence conversion to secondary oxidation products (Kaya *et al.,* 1993; Satue *et al.,* 1995). Kucuk and Caner (2005) studied the effect of PET packaging materials and the various keeping conditions on storage stability of sunflower oil. The results showed that PET packaging, presence of oxygen, light and storage period increased the peroxide value, free fatty acids and iodine value in addition to soap content compared with glass packing in the above mentioned conditions (Kucuk and Caner, 2005), in this case they leave a great impression on decreasing the oil storage stability.
The effect of different type of plastics (polyethylene terephthalate (PET), polyvinylchloride (PVC), polypropylene (PP) and polystyrene (PS) on the stability of olive, sunflower and palm oil were studied (Tawfik and Huyghebaret, 1999). The authors have concluded that the period and temperature of storage as well as packaging materials have significant effects on the stability of the above mentioned oils (Tawfik and Huyghebaret, 1999). Tawfik (2005) studied the oil absorption and the global migration of different types of plastic material (PET, PVC, PP and PS) into different vegetable oils (olive, sunflower and palm oil). The findings showed that the amount of overall migration from plastic packaging into a vegetable oil is determined by the type of plastic packaging and the kind of oil considered. Tawfik concluded that the chain length of the fatty acids and the degree of saturation clearly influence the oil absorption by polymers whereas the migration of components from plastic was not influnced that much (Tawfik, 2005).
The present study examined the effects of PET plastic pieces and various storage conditions on fatty acid profile and some quality factors. Hence, Fatty acid profile and quality factors such as peroxide value, free fatty acids and iodine value and induction period before and after storage of samples at 25 and 45℃ for 20 and 60 days in presence or absence of PET pieces in three types of common oils from Iran's market were investigated.

MATERIALS AND METHODS

Oils and PET bottles : Commercial sunflower, canola and blended oils (contains sunflower, soy and cotton seed oils)(amount of added B.H.T as antioxidant 100 mg/kg in all of the oils) and PET bottles were obtained from Savola Behshahr Co., Tehran. Iran. Chemicals solvents were purchased from Merck Co., Germany.

Sample preparation

After measuring a specific surface on the bottles, they were cut to14 pieces with the same size (surface of them are 6 cm^2). Pieces were placed in 250ml Glass vessels, the glass vessels were poured with oil up to 150ml volume of container, so that the test pieces were always remained well apart from one to another and immersed completely in each type of oil.

The glass vessels that contain tests pieces were stored at 25 and 45℃ for 20 and 60 days (Tawfik, 2005). Since all of the samples have been stored in a dark place and in sealed container (in glass vessels 250 ml) the effect of light and oxygen parameters in all specimens were similar. The temperature were controlled and the data recorded by data logger (LASCAR, England). Glass vessels containing oils only were placed in the same conditions and served as blank samples. Every treatment was performed in four replicates.

Chemical tests

By using rancimat system (Metrohm model 734, Switzerland) and AOCS (Cd 12b-92) the induction period test was done at 110 ℃ (Firestone , 1994).

The iodine value was calculated based on mathematical formula which presented in AOCS (Cd 1c-85), which directly calculated from the oil fatty acid profile (Firestone, 1994). In oxidative rancidity oxygen is taken by the oil with the formation of peroxide. The degree of peroxide formation (Peroxide Value) was calculated according to the AOCS (Cd 8-63) (Firestone, 1994). Determination of free fatty acids was done by AOCS (Cd 3d-63) (Firestone, 1994).

Statistical analysis

Experiments on each of samples were performed at four times. Two analyses were taken from the test samples at each specific time interval. Statistical analysis (Mean values and standard division were calculated at each time interval, so were analyzed by SPSS ver. 17 (SPSS Inc. Michigan Avenue., Chicago, USA) and Minitab ver. 11.12 (Minitab Inc., USA).

RESULTS AND DISCUSSION

Effects of time passing and raising of temperature caused breaking 2 cis double bonds and converting them to single double bond and without double bonds, Also, the conversations between different shape of geometrical isomerization such as Cis or Trans and local isomerization such Iso caused above mentioned alteration (Tarzi *et al*., 2006).

Since all of the samples have been stored in a dark place and in sealed container (in glass vessels 250 ml) the effect of light and oxygen parameters in all specimens were similar. Presence or absence of plastic pieces has no significant effect on fatty acids profile

According the results which is shown in Fig. 1, significant decrease ($P \leq 0.05$) was observed at induction period (IP) in the mentioned oils after storage for 20 and 60 days at 25 and 45℃. Among the oils, canola has maximum induction period because this oil contain a large amount of oleic fatty acid (mono unsaturated) and the blended oil has the minimum induction period because the oil contains different types of fatty acids such as poly unsaturated fatty acids. A significant difference ($P \leq 0.05$) at induction period was observed in the presence or absence of plastic pieces. These differences indicate the effect of plastic pieces on decreasing induction period and correlates with previous investigations (Huang *et al*., 1981; Kucuk and Caner, 2005). In the case of the blended oil, the significant decreasing in induction period after 60 days at 45°C has made the oil useless, with consider to blended oil standard (maximum authorized of induction period is 8 hours) (Institute of Iran Standard and Industrial Researches, 1981; Institute of Iran Standard and Industrial Researches, 1999; Institute of Iran Standard and Industrial Researches, 2007). The result shows that the canola oil has the maximum induction period in comparison with other oils.

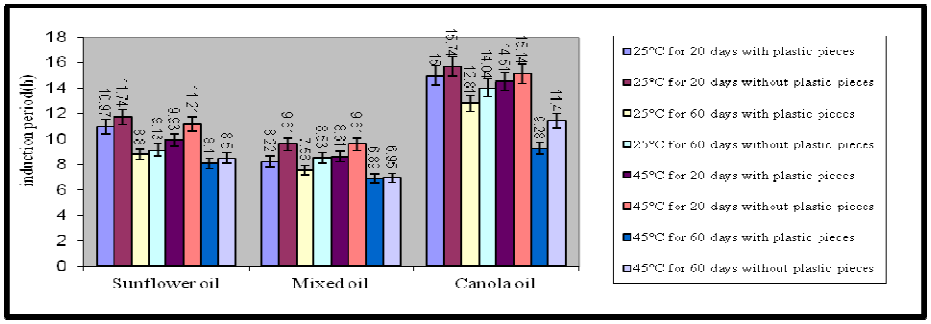

Figure 1. The induction periods of sunflower, blended (Mixed) and canola oils during different storage conditions

As shown in Fig. 2, sunflower and blended oils have increased significantly (P≤0.05) in peroxide value(PV) after 20 and 60 days at 25 and 45℃ and in the presence or absence of PET pieces but in canola oil only period and temperature has affected significantly (P≤0.05) in peroxide value. In blended oil that contains different types of fatty acids; peroxide value was formed quickly because of the large amount of poly unsaturated fatty acids. Peroxides in sunflower and canola oil was formed slower than blended oil due to the presence of high amount of natural antioxidants and mono unsaturated fatty acids like oleic acid, respectively (Kucuk and Caner, 2005; Kadivar *et al*., 2010; Cinquanta *et al*., 2001) but during storing and after decreasing efficiency of natural antioxidants and decreasing amount of mono unsaturated fatty acids the amount of peroxide value has increased in sunflower and canola oil in comparison with the initial oils. In presence and absence of plastic pieces, the sunflower and canola oil has increased significantly (p≤0.05) in peroxide value after 20 and 60 days at 45℃. Relatively, the PVs have been alte red at the end of storage period, and have been increased as a result of releasing pro oxidant compounds such as aldehydes from plastic pieces. Moreover, it has been stimulated by increasing temperature. This result was confirmed by previous investigations (Huang *et al*., 1981; Frankel, 1991; Kucuk and Caner, 2005; Ghasemnezhad and Honermeier, 2007). Maximum authorized value of peroxide in National standard of Iran for sunflower, canola and blended oils are 2.5, 2.0 and 5.0 meq/kg, respectively, so based on these values, sunflower oil which stored at 45℃ in both of storage periods, canola oil which stored at 45℃ for 60 day s and blended oil that stored at 45℃ for 20 days with plastic pieces, 60 days with and also without plastic pieces became useless (Institute of Iran Standard and Industrial Researches, 1981; Institute of Iran Standard and Industrial Researches, 1999; Institute of Iran Standard and Industrial Researches, 2007). Results show that canola oil has the minimum PV in comparison with other oils at the end of storage period.

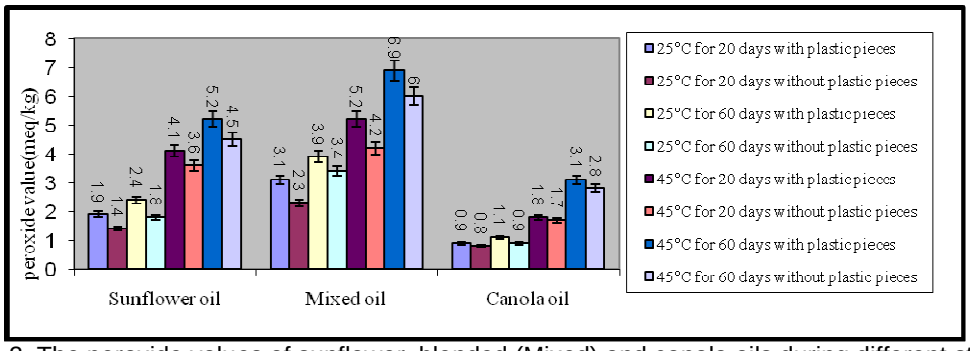

Figure 2. The peroxide values of sunflower, blended (Mixed) and canola oils during different storage conditions

The Iodine value (IV) is decreased during the storage period as shown in Fig. 3. The amount of unsaturated fatty acid has a direct effect on IVs. As the result existence of poly unsaturated fatty acids in the blended and sunflower oil, The IVs in sunflower and blended oil significantly declined (P≤0.05) after 60 days at 45℃ and in presence or abse nce of PET

pieces. Slight changes after decreasing of iodine value in canola oil were observed. The Results correlates with the previous investigations (Tawfik and Huyghebaret, 1999).

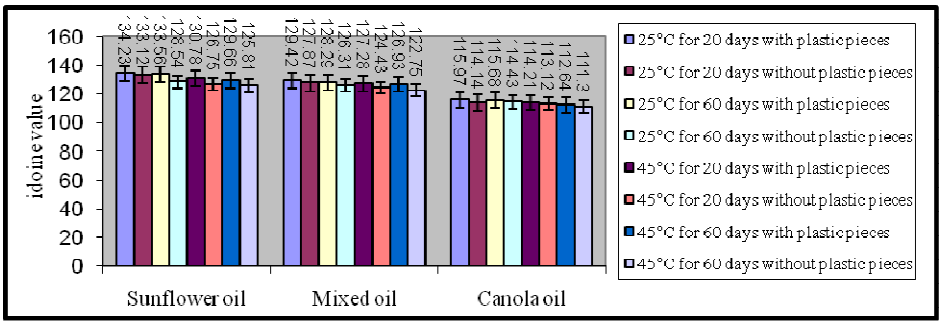

Figure3. The iodine values of sunflower, blended (Mixed) and canola oils during different storage conditions

Amounts of free fatty acids (FFA%) during different conditions of storage were shown in Fig. 4. It is well accepted that during storage period, partial hydrolysis of oils has taken place, thus free fatty acid content were increased. There was a significant increase (P≤0.05) in the FFA% among the storage for 20 and 60 days at 25,45℃ in mentioned oils which shows the effect of temperature and time on forming free fatty acids, and also confirm the previous investigations (Sharma *et al*., 1990; Frankel, 1991; Ghasemnezhad and Honermeier 2007). FFA% had increased significantly (P≤0.05) in presence or absence of the plastic pieces in the oils. Releasing lower molecular acids from plastic pieces which are stimulated by increasing temperature cause the Increasing of FFA %. In National standard of Iran, the maximum permissible level of free fatty acids in sunflower, canola and blended oil are 0.2, 0.2 and 0.1%, respectively, thus the blended oil which had been stored at 45^0C for 20 and 60 days with and without plastic pieces was deteriorated (Institute of Iran Standard and Industrial Researches, 1981; Institute of Iran Standard and Industrial Researches, 1999; Institute of Iran Standard and Industrial Researches, 2007). It is necessary to be noticed that the presence of free fatty acids increases the hydrolysis which leads deterioration of oils and make them unsuitable for human consumption. Results show that the canola oil has the maximum level of free fatty acids in comparison with the other oils.

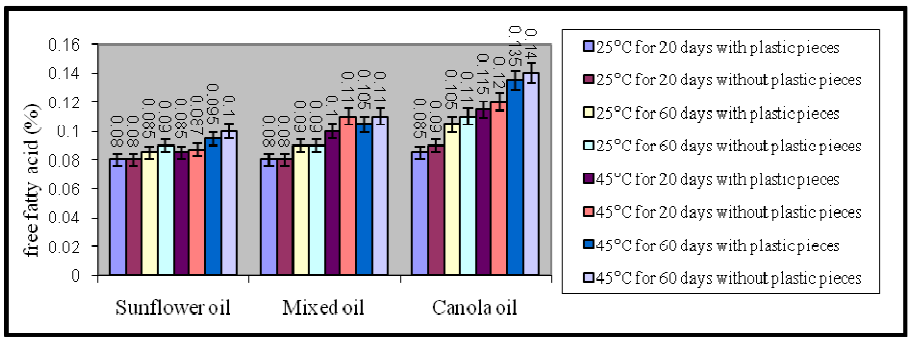

Figure 4. The amount of free fatty acids of sunflower, blended (Mixed) and canola oils during different storage conditions

CONCLUSION

On the examination of quality factors such as free fatty acids, peroxide value, induction period and iodine value, some results clearly emerged. Increasing storage period and temperature and presence of PET pieces has some effects on FFA%, PV, induction period and Iodine value. It is concluded from this study that the stability of vegetable oils is dependent on the type of oil and its initial physical and chemical properties, time and temperature of storage and the type of packaging (PET and Glass). In addition increasing

storage temperature and time accelerated the deterioration and limited the stability of vegetable oils. Results show the quality of oil has been decreased after the storage at high temperature (45℃) and long time of storage. So for preventing of deterioration, the oils which were packed in PET bottles should be stored at a temperature lower than 25℃. If the temperature is raised (T<45℃) the shelf life of pr oduct should be limited. Difference between these effects in the mentioned oils can be explained by their nature, initial physical and chemical properties of oils. Oil stability can be enhanced by selection of a suitable package.

ACKNOWLEDGMENTS

The authors thank Dr. Bahmanei and Mr. Shoaei for research help. This study was supported by the Food and Drug Control Laboratories (FDCLs) of Iran.

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A NEW METHOD FOR DETERMINATION OF BISPHENOL A DIGLYCIDYL ETHER (BADGE) AND ITS DERIVATIVES IN CANNED OILY FOODS

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ABSTRACT: Bisphenol A diglycidyl ether (BADGE) and its derivatives are determined in canned oily foods from Iran's market using a new simplified extraction method. Samples are extracted with methanol, back extracted with methanol, and were analyzed by using reversed –phase high performance liquid chromatography (RP-HPLC) with fluorescence detection (FLD) and excellent validation data were obtained. Whereas detection limit for BADGE.2H2O was detected in 0.04 ppm. Also the present of recovery for BADE and its derivatives in comparison to previous investigations is suitable. This method can detect the amount of BADGE and its derivatives in low concentration. From 57 analyzed cans, containing various fatty tuna fish products, BADGE.HCL, BADGE.H_2O.HCL and BADGE.H_2O were not detected in any samples. However, BADGE, BADGE.2H2O and BADGE.2HCL were detected in samples but the amounts of specific migration of BADGE and its derivatives from metal cans into the samples conform to European Community legislation (EC 1895/2005 that determined specific migration limits (SML) for sum of the BADGE and its hydrolyzed derivatives is 9 mg/kg and Sum of chlorinated derivatives is, 1 mg/kg), which confirm the safety of these cans container that were used for packaging oily foods in Iran.
Key words: *BADGE, HPLC, Specific Migration, canned oily food*

INTRODUCTION

Nowadays food packaging is one of the most important fields in food industry and also has certain effect on food safety issue. Most of cans which use for preserving food are coated by interior lacquer based on epoxy resins to have a barriers role between the food or beverage and the metal surface of the cans for presenting good condition for products.
Bisphenol A diglycidyl ether (BADGE) is the condensation reaction product of one mole of Bisphenol A (BPA) with two moles of epichlorohydrin. BADGE was used as a starting substance or stabilising components for epoxy resins (Suarez, *et al*., 2000).
BADGE hydrolyses occurs in contact with aqueous and acidic food and it may be convert to forms of mono- and dihydrolysed products (BADGE.H2O and BADGE.2H2O) of BADGE or chlorinated products (BADGE. HCL, BADGE.2HCL) or BADGE H_2O HCL, that can be migrate from can lacquers into food stuff (Fig .1) (Hammarling, *et al*., 2000; Forrest, *et al*., 2005; Satoh, *et al*., 2004). BADGE may break down and stability of BADGE depended on condition and environment of storage. Also the natural of food has effect of this stability (Poustkova, *et al*., 2004).
The epoxy resins are alkylating bifunctional agents and they have specific cytotoxic actions in tissues with high rates of cell division vitro assays using different endpoints. In the case of hydrolysis products and BADGE chlorohydrin no data exists about their genotoxicity (Suarez, *et al*., 2000). BADGE cause disfunctionality of sex (female) hormones and also is of concern due to its teratogenicity and carcinogenicity (Rauter, *et al*., 1999; Peterson, *et al*., 2003; Kang, *et al*., 2006; Rykowska, *et al*., 2006). With consider to this issue there is a lack of

information concerning BADGE and its derivatives migration from can coatings into canned sea food in oil which has a high level of consumption in Iran market.
In 1996 in Switzerland market, BADGE was detected in oil that extracted canned fish. They had a higher level of the maximum permissible amount of Switzerland legislation and removed of the consumption market (Shafagh Gilani, *et al*., 2007).
Rauter, *et al*. in 1999 determined bisphenol A diglycidyl ether (BADGE) and its hydrolysis products in canned oily foods from the Austrian market by using a new simplified HPLC method. Samples are extracted with pentane, back extracted with methanol, and finally dissolved in the mobile phase (cyclohexane/*tert*–butyl methyl ether). Separation is performed on a normal phase HPLC column using fluorescence detection. Results showed that from 67 analyzed cans, containing various fatty meat or Fish products, 16% were above the maximum quantity of 1 mg/kg tolerated by the European Community, 45% were in the range between 0.1–1 mg/kg, 24% between 0.02 and 0.1 mg/kg, and in 15% the BADGE concentrations were below the detection limit of 0.02 mg/kg. The hydrolysis product BADGE.H2O was not detected in any sample, whereas BADGE.2H2O was found in some samples up to a concentration of 0.5 mg/kg (Rauter, *et al*., 1999). BADGE has been examined in canned fish in oil. Cans and lids were extracted by acetonitryl separately. BADGE extracted of fish with hexane and re-extracted with acetonitryl. The method of analysis was by RP-HPLC with fluorescence detector. Finally BADGE was detected in 12% of fishes, 24% of the cans and 18% of the lids (Theobald, *et al*., 2000). Migration of BADGE and its derivatives in canned foods have been determined by Hammarling, *et al*. in 2000. In this research the greatest amount of BADGE was found in canned fish in oil (5.1 mg/kg). BADGE was also found up to 1.1 mg/kg in the fish in tomato sauce (Hammarling, *et al*., 2000) Leepipatpiboon, *et al*. in 2005 determined bisphenol-A-diglycidyl ether,bisphenol-F-Diglycidyl ether, and their derivatives in oil-in-water and aqueous-based simultaneously in canned foods which was applied by high-performance liquid chromatography with fluorescence detection. The method detection limits range 0.72–4.20 ppb and the method quantitation limits range 2.40–14.85 ppb, respectively (Leepipatpiboon, *et al*., 2005). Cabado, *et al*. in 2008 evaluated kinetics migration of BADGE and BFDGE from varnish into canned products (sardines, tuna fish, mackerel, mussels, cod and mackerel eggs) by HPLC in 70 samples after 6, 12 or 18 months of storage. Results of this research showed that there is no migration of BADGE in tuna fish, sardines, mussels or cod. However, migration of BFDGE occurs in all species, in a storage time-dependent way and content of fat, although migration of these compounds is not affected by sterilization conditions (Cabado, *et al*., 2008).
Specific migration limit (SML) for sum of BADGE, BADGE. H_2O and BADGE.$2H_2O$ is set to 9 mg/kg and for sum of BADGE. HCl, BADGE .2HCl and BADGE H_20 HCl is set to 1mg/kg in the legislation (European Standard, 2005).
The main purpose of this study is determination of migrated BADGE and its derivatives in canned oily foods by simplified extraction and HPLC Method.Also, comparison of final result with European legislation has been done for confirmation safety of used lacquer in can.

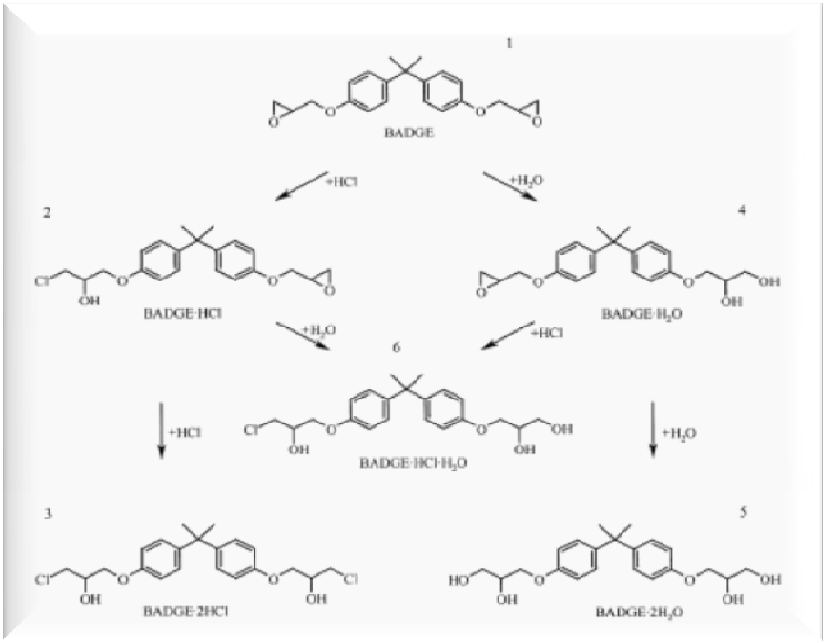

Fig 1. Bisphenol A diglycidy ether (*BADGE*) and its reaction products (Peterson, *et al*., 2003)

MATERIAL AND METHODS

Materials: Standards and reagents

All standards of BADGE and its derivatives (BADGE.H20, BADGE.2H_2O, BADGE.HCL, BADGE.2HCL, and BADGE.H_2OHCL) were purchased from Fluka chemical Co., Swiss. Stock solutions of BADGE and its derivatives were made in methanol at a concentration of 100µg/ml and were stored at 4 $^{\circ}C$ .Intermediate solutions of BADGE and its derivatives were prepared at a concentration of 10 µg/ml in methanol. HPLC-gradient grade water and methanol solvents were purchased from Merck Co., Germany

Samples

The experiments have performed on 57 canned oily foods (tuna fish in oil) that purchased and analyzed at least 6 months after producing.

Extraction process

The whole content of each can include the solid material and the oil, was homogenized using a laboratory Mixer. 2.5 g of this homogenate was weighted accurately into a 15 ml CellStar Tube, 5 ml methanol was added and the tube was sealed and properly shaken on a Vortex mixer. Afterwards centrifuged and the supernatant was transferred into a second tube. The extraction was repeated twice. The collected methanol fraction was evaporated under nitrogen stream up to approximately 4ml, after adding 1ml distillated water Centrifuged in refrigerate at 3500 rpm in -2 ℃ for 10 min in orde r to separation, extracted samples were filtered through a micro filter (whatman, diameter 13 mm, pore size 0.45µm) then analyzed by HPLC with Fluorescence detector for determination of migrants.

Measurements of BADGE and its derivatives by HPLC

Standards and the content of these monomers in samples were separated and quantified by using a HPLC system (Agilent 1200, Germany) equipped with an Agilent G1311A quaternary pump , an Agilent G1315A Florescence Detector (FLD) and C18 Agilent column (150mm, 5 µm particle diameter, and 4.6mm internal diameter was used). The column temperature was kept at 30℃ by using a column oven . The used wavelengths for detection of monomers were 225 nm (excitation wavelength) and 305 nm (emission wavelength). The

binary gradient conditions were used: H2O / Acetonitrile (60:40v/v) to H2O / Acetonitrile (40:60 v/v) and Flow Rate: 0.75 to 1.5 ml/min were established. The volume of injection was 5 µl as illustrated in table 1. (Mousavi Khaneghah, *et al.,* 2012).
A mix stock standard solution (10 ppm) were prepared from BADGE and its derivatives in methanol and stored in the dark place at refrigerator temperature. Calibration standards solutions were prepared on the day of use at levels 5, 2, 1, 0.5, 0.25; 0.125and 0.0625µg/ml of stock standards solution and 5 µl of each standard were injected to HPLC at three times**.** Standard curves for all standards were plotted by injection 7 concentration of standard and peak area responses are obtained. A standard graph was prepared by plotting concentration versus area (Mousavi Khaneghah, *et al.,* 2012).

Table 1. Conditions of HPLC System

Mobile phase	**ACN/H2O(40/60)- ACN/H2O(60/40)**
Flow rate	**0.75-1.5ml/min**
Interior pressure	**72-105 bar**
Amount of injection	**5µl**
Column type	**C18Silica Agilent**
Particle size	**5 µm**
Column length	**150 mm**
Column diameter	**4.6 mm**
Oven temperature	**30 C**
Run Time	**12 min**
Type	**Gradiant**
pump	**Quaternary pump Agilent G1311A**
Oven	**Agilent G1316A**
Detector	**(F.L.D)Agilent G1321A Fluorescence Detector**
Solvent Degasser	**Agilent G1322A**
Syringe	**Agilent Syringe 50 µl**
Injector	**Agilent G1328B**

RESULTS AND DISCUSSION

Results from method validation

Recovery studies were carried out by spiking selected samples of homogenized Tuna Fish with mixed standard solution (mix of BADGE and its derivatives) at four different concentrations (0.25, 0.5, 2 and 4ppm). The spiked samples as well as controls were analyzed in 3 replicate experiments. The recoveries were calculated by using standard calibration curves. The detection limit (LOD) and quantitation limit (LOQ) are defined as the amount of analyte in standard solutions that yields an instrumental signal significantly different from the blank or background signal which equals to 3 and 9, respectively. Depending on the spiked concentration, the recovery rates were between 70% and 120% and the reproducibility for the whole determination procedure was less than 20%.The correlation coefficient for the linear regression curve was found more than 0.994 in the range of 0.0625 – 5 µg/ml. Table 2 summarizes R^2, Recovery, LOD and LOQ values of individual compounds and clearly indicates that the analytical method has excellent sensitivity.

Table 2. Results of method validation

Type of Analyte	***R^2***	***LOD****	***LOQ****	***Recovery*****	***CV*****
BADGE	0.999	0.083	0.250	74 -114	4.81-17.16
BADGE.H_2O	0.997	0.083	0.250	77 - 98	3.90-10.25
BADGE.2H_2O	0.994	0.040	0.120	83 - 98	5.6 -11.14
BADGE.HCL	0.998	0.160	0.500	89 - 100	0.46- 5.22
BADGE.2HCL	0.996	0.083	0.250	95 - 105	5.86-19.70
BADGE.H_2O.HCL	0.995	0.050	0.150	99 - 116	0.83-7.63

***Per ppm** ****Per percent**

Results from various samples

Altogether, 57 canned oily food, were analyzed for their content of hydrolyzed and chlorinated derivatives of BADGE. Result shows that the amount of migrated Compounds (hydrolyzed and chlorinated derivatives) into fish from Can metal coating during this experiment were lower than EC Nº1895/2005 legislation (with maintained specific migration limits for sum of the BADGE and its hydrolysis derivatives is 9 mg/kg and Sum of chlorinated derivatives is, 1 mg/kg) as observed in Table 3, so can containers can be considered to be in compliance with EC regulation. No BADGE.HCL, BADGE.H_2O.HCL and BADGE.H_2O were detected in any samples whereas BADGE.$2H_2O$ was found up to 41.22% in samples. Also BADGE up to 31.57% and BADGE.2HCL up to 5.26% were detected in samples. From all samples were analyzed 67.5 % of hydrolyzed derivatives and 5.2 % chlorinated derivatives of BADGE were above quantification limit. Also the chromatograms of HPLC for one of the samples and standards have been shown in fig 2 and fig 3.

Table 3. The average of migrated derivatives in all samples

Analytes	Amount (mg/kg)
Hydrolyzed Derivatives	0.7619
Chlorinated Derivatives	0.0301

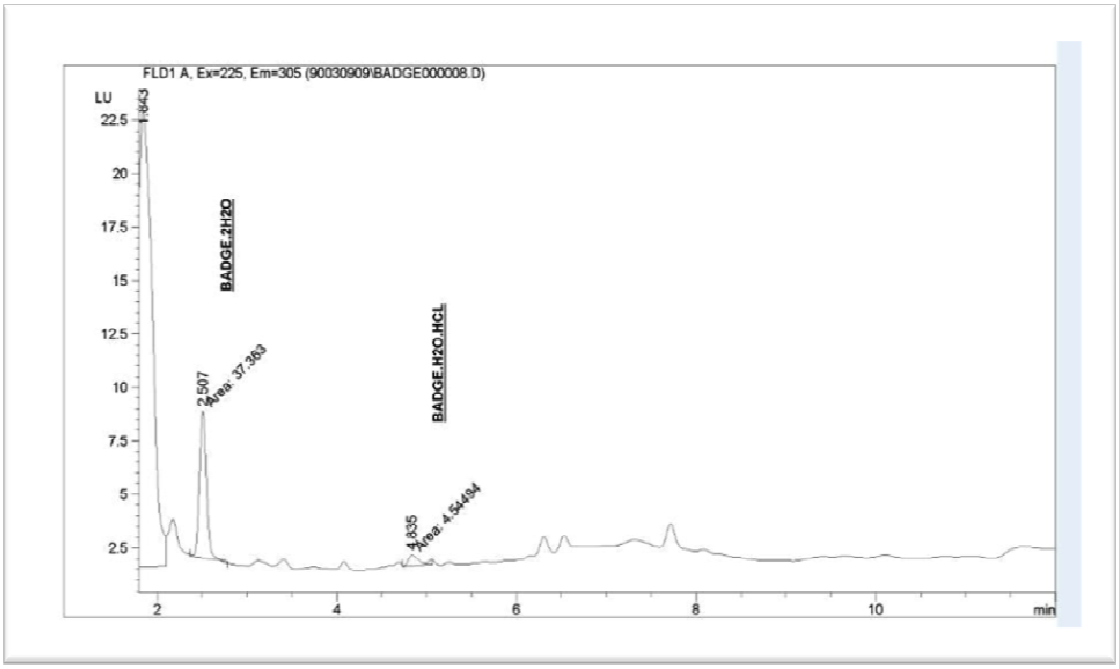

Fig 2. HPLC chromatogram for one of the samples

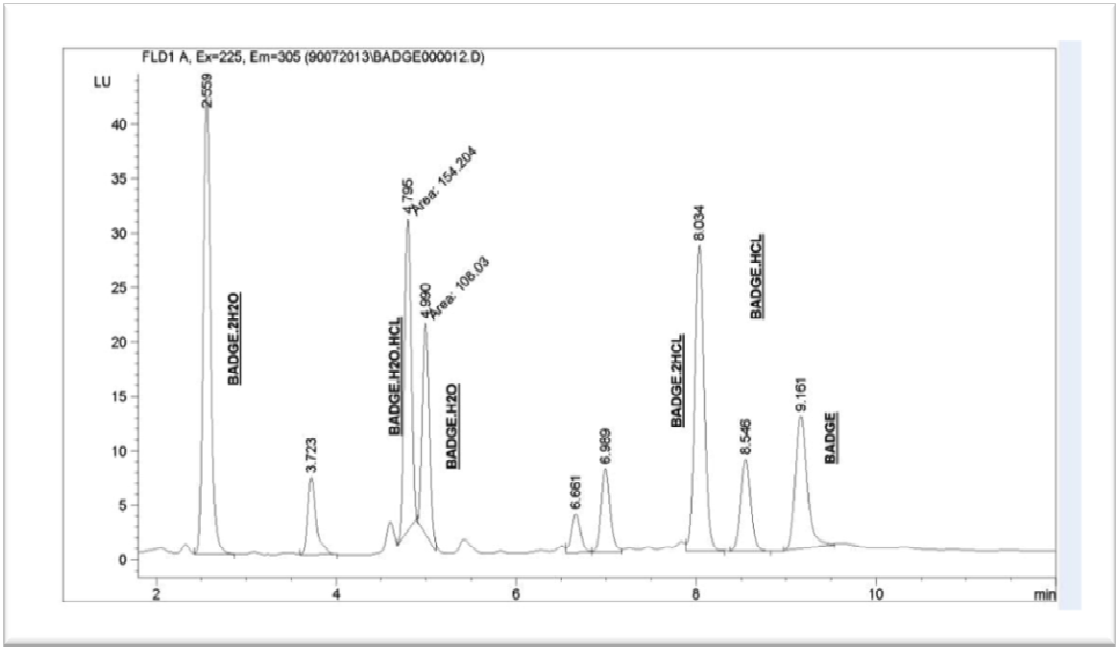

Fig 3. HPLC standard chromatogram for mixture of BADGE and its derivatives in concentration of 1 ppm

CONCLUSIONS

HPLC method with Fluorescence detector was used for determination of migration of BADGE and derivatives from can containers into canned oily food. This study was carried out for investigation of quantify migration of hydrolyzed and chlorinated derivatives of BADGE in canned oily food. Extraction with centrifuge under cold condition (-2 °C) provides a rapid and accurate method for determination of BADGE and its derivatives by HPLC with fluorescence detection especially decreased run time in comparison with previous investigations that have been performed on canned food (Rauter, *et al*., 1999;Hammarling, *et al*., 2000;Cabado, *et al*., 2008).

More studies should be done on migration of BADGE and its derivatives at different types of canned food. It is necessary to control and improve the distribution chains and the conditions of storage to ensure the safety of products.

This method in this study can detect the amount of BADGE and its derivatives in low concentration. Whereas detection limit for BADGE.$2H_2O$ was detected in 0.04 ppm. Also the limit of recovery for BADE and its derivatives in comparison to previous investigations is suitable (Rauter, *et al*., 1999; Hammarling, *et al*., 2000; Cabado, *et al*., 2008).

Results showed that the BADGE.HCL, BADGE.H_2O.HCL and BADGE.H_2O were not detected in any samples. However, BADGE, BADGE.2H2O and BADGE.2HCL were detected in samples but the amounts of specific migration of BADGE and its derivatives from metal cans into the samples conform to EU legislation (EC 1895/2005 that determined specific migration limits (SML) for sum of the BADGE and its hydrolyzed derivatives is 9 mg/kg and Sum of chlorinated derivatives is, 1 mg/kg), which confirm the safety of these cans container that were used for packaging of canned oily food in Iran's market.

ACKNOWLEDGEMENTS

This study was supported by Food and Drug Control Labs, Ministry of Health of Iran. The Authors are grateful staff of Packaging and Toxicology Labs, Food and Drug Control Labs for their helpful cooperative during this project.

Authors have special thanks to Dr Nemati and Mrs Fathi as their helpful guides.

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WOUND-INDUCED RESPONSES DURING LOW TEMPERATURE STORAGE OF SHREDDED CARROT

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ABSTRACT: Fresh-cut shredded carrot processing involves wounding operations (peeling and cut) which lead to fast quality deterioration (physiological and microbiological). However, advantages can be drawn from the use of wounding due to the related effect of phenolic synthesis induction. Phenolic compounds are known to be present in higher concentrations in carrot peripheral tissues (peels) than in the internal ones. In view of the processing diagram to fresh-cut carrot production it is of utmost importance to characterize the peeling operation effects in regard to the balance between phenolic losses and synthesis during storage. This study aimed to evaluate the effects of wounding intensity (peeling and shredding) on the total phenolic content (TPC) of *cv*. Navajo carrot during storage (7 days/5 °C). Higher phenolic contents were observed (≈40%, $p<0.05$) in unpeeled carrot samples, demonstrating the significant contribution of peels to the carrots total phenolic content. Wound-induced responses were dependent on wounding intensity and storage time. By the end of storage, TPC levels of 246.4 and 155.3 mg CAE/100 g were found in unpeeled and peeled shredded samples, respectively, corresponding to increases in TPC when compared to raw material ($p<0.05$; 128.6 mg CAE/100 g). Peel removal did not compromise wound-induced responses. These results are of great importance to the fresh-cut technology since peel removal prevents accelerated microbial decay while allowing the production of high quality products with expectable longer shelf-lives.
Key words: *Shredded carrot; Wounding intensity; Stress; Phenolic content*

INTRODUCTION

The fresh-cut fruits and vegetables (F&V) (washed, cut, packaged and refrigerated products) market is the fastest growing segment on the F&V market in the European Union (EU) and its success is mainly due to their convenience, freshness and associated health benefits (Ragaert *et al.*, 2004). Shredded carrot is an important fresh-cut commodity due to its pleasant flavour and nutritional benefits as well as to its versatility of use (Alasalvar *et al.*, 2001).

Minimal processing operations damage carrots tissue integrity and triggers deteriorative processes which lead to increases in microbial and non-microbial spoilage during storage (Jacxsens *et al.*, 2003). However, the mechanical and physical stresses imposed to carrots during minimal processing operations are elicitors of wound-induced phenolics and can be used to increase the antioxidant potential of shredded carrot.

Phenolic accumulation is a usual wounding stress response, part of the plants defence mechanism through activation of the phenylpropanoid pathway (Dangl *et al.*, 2000), and is dependent on several factors such as wounding intensity (Heredia & Cisneros-Zevallos, 2009), initial concentration (Reyes *et al.*, 2007) and storage conditions (Jacobo-Vellasquez and Cisneros-Zevallos, 2009). This defence system triggers the activation of phenylalanine ammonia lyase (PAL) and is followed by the synthesis of protective phenolic compounds to reduce water loss or pathogen attack (Rhodes and Wooltorton, 1978).

Plant peripheral tissues provide a primary defence barrier to exogenous stresses (both biotic and abiotic) and are richer in phenolic compounds (Hutzler *et al.*, 1998). It is known that phenolic distribution varies significantly among the different carrot tissues (peel, phloem and xylem) where, according to Zhang and Hamauzu (2004), peels provide about 50% of the total amount of phenolic compounds present in the root. Peel removal is part of the minimal processing operations for shredded carrot production since it provides benefits that include a significant reduction of the initial epiphytic microflora and improves products sensory quality. Although several studies can be found about the effects of wounding intensity over phenolic accumulation in unpeeled shredded carrot, information about the effect of peel removal over the wound-induced stress responses is scarce. For this reason, the aim of this research was to quantify the effect of peeling and shredding on the total phenolic content of *cv*. Navajo carrot during low temperature storage (5 C).

MATERIAL AND METHODS

Plant Material and Processing

Carrots (*Daucus carota* L. *cv.* Navajo) were obtained from a local market (HEB, Grimmway Farms, Bakersfield, CA), sorted and washed with water to remove excess dirt. Carrots were divided into 4 groups: *I)* Whole unpeeled carrots; *II)* Whole peeled carrots; *III)* Unpeeled shredded carrot and; *IV)* Peeled shredded carrot. Carrots were shredded using a West Bend® High Performance Food Processor (The West Bend Co.; West Bend, WI). After preparation, samples were placed separately in 4-L clear glass jars (closed and vented every 8 h to avoid CO_2 accumulation) and stored for 7 days at 5 °C. Total phenolic determination was performed using five replicates for each sample type at days 0, 3 and 7 of storage.

Quantification of total phenolic content

Carrot tissue (5 g) was homogenized with methanol (20 mL) using an Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, NC) and centrifuged at 29000 g for 15 min at 4 °C. The clear supernatant (methanolic extract) was used for total phenolic content determination using the method described by Swain and Hillis (1959). Methanolic extracts (15 µL) were diluted with nanopure water (240 µL) in a 96-well microplate, followed by the addition of 0.25 N Folin-Ciocalteu reagent (15 µL). The mixture was incubated for 3 min, and then, 1 N Na_2CO_3 (30 µL) was added. The final mixture was incubated for 2 h at room temperature in the dark. Spectrophotometric readings at 725 nm were collected using a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). Total phenolic content was expressed as mg chlorogenic acid equivalents/100 g of fresh tissue.

Statistical analysis. Data from the trial was subjected to analysis of variance (One-way ANOVA) using the Statistica™v.8 Software from Statsoft (StatSoft Inc., 2007). Statistically significant differences ($P<0.05$) between samples were determined according to Tukey Honestly Significant Difference (HSD) test.

RESULTS AND DISCUSSION

Total phenolic content of whole and shredded carrot, with and without peels, after processing is shown in table 1. According to the higher levels ($p<0.05$) of total phenolics assessed in both unpeeled samples compared to the corresponding peeled ones, it can be concluded that the peel accounts for about 40% of cv. Navajo carrot total phenolic content. Zhang and Hamauzu (2004) also found that even though carrot peel accounted for only 11.0% of carrots fresh weight, it provided about 54% of carrots total phenolic content in Chibagosun and Hitomigosun varieties.

Table 1. Changes in total phenolic content (TPC) after wounding (day 0).

Shredding	Peeling	TPC (mg CAE/100 g)	TPC variation (%)
Whole	With peel	128.6^{b}±17.1	42.81
	Without peel	55.1^{a}±4.5	
Shredded	With peel	111.8^{b}±5.5	44.61
	Without peel	49.9^{a}±3.1	

Results are given as mean ± standard deviation (n=5). In the same column, different letters indicate significant differences (HSD test, at p≤0.05).

Just after processing and despite the peeling effect, when comparing whole and shredded samples it was found that shredding operation itself (table 1) did not introduce any change in TPC levels ($p>0.05$).

Phenolic content of whole samples with peel (raw material) remained relatively unchanged ($p>0.05$) during storage, ranging from 128.6 to 142.6 mg CEA/100 g (Figure 1). The peeling effect in carrot (whole samples without peel – mild wounding), induced phenolic synthesis since a significant increase in TPC of 1.3 times from day 3 to 7 (63.8 to 85.5 mg CEA/100 g, $p<0.05$) was found.

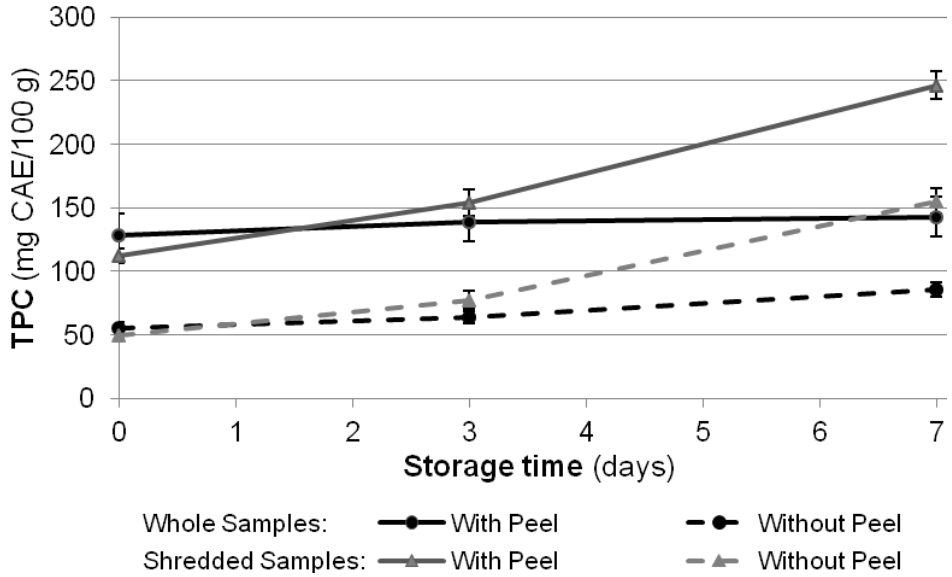

Figure 1. Effects of peeling and shredding on the total phenolic content of carrots during storage (7 days at 5 °C). Results show means of five replicates and their standard deviation.

During storage, both shredded samples had significant increases in TPC levels ($p<0.05$), showing that wound-induced phenolic accumulation is also dependent on storage time in accordance with other works (Reyes *et al.*, 2007; Heredia and Cisneros-Zevallos, 2009). The shredding effect accounted for increases in TPC levels of 2.2 and 3.1 times in unpeeled and peeled samples, respectively. This higher wounding intensity induced higher phenolic synthesis rates than peeling. This points out to the wounding intensity effect over the phenolic synthesis rate and accumulation which is in accordance to the findings of Heredia and Cisneros-Zevallos (2009). The authors found that phenolic synthesis followed a grading order from sliced, pie-cut and shredded carrot tissues while intact tissues (whole and unpeeled) had no significant change in TPC during storage (12 days, 15 °C).

Our results show that peel removal does not compromise wound-induced responses and that phenolic synthesis was independent of the initial TPC level. It is noteworthy from the fresh-cut quality standpoint that, during storage, peeled shredded samples TPC levels surpassed ($p<0.05$) the raw material level (155.3 *vs.* 128.6 mg CAE/100 g).

CONCLUSIONS

Wound-induced responses were dependent on both wounding intensity and storage time but independent on peel presence. Despite initial losses in phenolic content due to peel removal (up to 40%), peeled shredded carrot surpassed the raw material level by 27% during storage. These results are of great importance to the fresh-cut technology since peel elimination prevents accelerated microbial decay while allowing the production of high quality products with expectable longer shelf-lives.

ACKNOWLEDGEMENTS

The author Carla Alegria acknowledges financial support to Fundação para a Ciência e a Tecnologia (Portugal), via a Doctoral fellowship (SFRH / BD / 62211 / 2009).

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CHARACTERISTICS OF PACKAGING MATERIALS FOR SPECIFIC PACKAGING CONDITIONS OF MEAT AND OSMOTIC DEHYDRATED MEAT

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ABSTRACT: Packaging necessary trace food products. Packaging materials with different properties affect the quality and sustainability of the meat and meat products. Consumer's interest has been increasing in natural and quality preserved food products that are not chemically treated. Improvement of the protective effect of applied packaging materials can be achieved by selecting appropriate conditions of packaging (vacuum, modified atmosphere and the latest trend is active packaging). The objective of this study was to review the possibility of application of different materials through examination of their mechanical, barrier and structural characteristics. This paper presents comparative results related to combinations of materials usually used in meat industry, obtained by different methods of production (laminating, extrusion laminating and co-extrusion). The results showed that monomaterials pass their good properties on multilayer materials. Multilayer materials have good characteristics and as such they meet the requirements of specific packaging conditions. These materials provide preservation of the protective atmosphere inside the packaging, thus contributing to the quality and sustainability.
Key words: *packaging materials, packing, characteristics, meat*

INTRODUCTION

The quality of packaged foods is greatly influenced by the properties of packaging materials. These vary depending on factors including the type of material, use of additives and method of manufacture. For instance, the properties of plastic films are mainly dependent on their composition, crystallinity and morphology (Sebranek and Houser, 2006). In meat industry most commonly used material are PA (polyamid) / PE (polyethylene) which could contain barrier layers: etilen-vinil-acetat (EVA), etilen-vinil-alkohol (EVOH), poliviniliden-hlorid (PVDC) and others (Lazić et al., 2002). Traditionally, the plastic films used for vacuum and MAP were developed to improve effectiveness in their gas and moisture barriers, shrinking properties, sealing characteristics, cook-in and retort capability and a variety of print and color options (Sebranek and Houser, 2006).
Modern meat packaging techniques are intended to maintain microbial and sensory quality of the product (Seydim et al., 2006; Fernandez-Lopez et al., 2008). This can be achieved by manipulation of the meat microenvironment (Hotckiss, 1988). Vacuum and modified atmosphere packaging (MAP) conditions are used in the food industry to extend the product shelf-life. MAP can be classified into two main categories, namely, low oxygen modified atmosphere (including vacuum packaging, CO_2 and N_2 gas flushing) and high oxygen modified atmosphere (Robertson, 1993). Modified atmosphere packaging has led the evolution of fresh and minimally processed food preservation, especially in meat and meat products for the past two decades. It was found that the extension of shelf life of meat samples depended on the packaging conditions and augmented in the order: air < vacuum pack < gas mixture 40%CO_2/30%N_2/30%O_2 < gas mixture 80%CO_2/20%air < 100% CO_2 (Skandamis and Nychas, 2002). Modified atmosphere packaging (MAP) using a high carbon dioxide (CO_2) environment is an effective means of prolonging microbial shelf-life of meat during extended storage (Sørheim and Nissen, 1999).

When the packaged meat is exposed to high O_2 concentration, growth of aerobic microorganisms, and oxidation of lipid and myoglobin are accelerated. Therefore, the use of gas barrier film to restrict the entry of O_2 through packaging material has been abundantly reported and widely commercialized in the industry (Kotzekidou and Bloukas, 1996). For instance, it was reported the shelf-life of MA packaged meat increased by 10–15% when using a barrier film with an O_2 permeability below 2 cm^3/m^2/day/atm (Gill and Molin, 1991).
Barrier films with O_2 permeability less than 100 cm^3/m^2/24 h/atm (at 23 ℃ and 0% rh) are generally being used for vacuum packaging or MAP of meat in the industry. The average O_2 permeabilities of polyamide (PA)/polyethylene (PE) films and polyvinylidene (PVDC)/EVA (ethylene vinyl acetate) copolymer films were 48.8 and 14.0 cm^3/m^2/day/atm, respectively. In order to maintain the gas composition inside the MAP over the storage period as constantly as possible, sheet thickness for the MAP tray should be at least 1–2 mm, and CO_2 and water vapor permeabilities should be lower than 65 cm^3/m^2/day/atm and 645 g/m^2/day/atm, respectively (Smith, 2001). In plastic films, CO_2 has 4–5 times and 13 times greater permeability than O_2 and N_2, respectively, because the solubility coefficient of CO_2 is much greater than the other gases (Robertson, 2006). Into commonly used barrier layers (PA, PETP and PVC), EVOH (ethylene vinyl alcohol), PVOH (polyvinyl alcohol) or PVDC are embedded in the multilayered structure by lamination or coextrusion (Lange and Wyser, 2003) of which O_2 permeabilities are normally less than 1 cm^3/m^2/24 h/atm (Stiles, 1990). In order to get an absolute barrier, very thin vacuum metalized aluminum is coated to the PETP layer. These days, consumers and retailers are more likely to demand flexible transparent barrier materials. In this regard, the disadvantages of vacuum metalized aluminum film are its lack of transparency and its inability to be used for microwaving (Lee, 2010).
There are two very popular and widely spread formats of packaging. These are high oxygen/barrier tray/barrier lid and high oxygen/tray/barrier over wrap. High oxygen/barrier tray/barrier lid consists of a clear or colored barrier lined tray (PS, PP, PE) that is paired with a clear or printed barrier film. This style of package normally has a 1:1 headspace ratio and contains an atmosphere of 80% O_2 and 20% CO_2. The 1:1 ratio is needed to give the product a minimum of 55% oxygen throughout the shelf life, which was found to provide optimal color life (Jakobsen and Bertelsen, 2000). The O_2 reacts with the meat to extend the oxymyoglobin state while the CO_2 acts as bacteriostatic agent (Kropf, 2004). The advantages of this type of product is that everything can be done at the packing plant and all that needs to be done by the processor is to place the pre-priced/pre-labeled package into the case. The disadvantage is that by marketing the product in the oxymyoglobin state the quality life of the product is limited to about 10–12 days for ground beef and 12–16 days for whole muscle. Lipid oxidation tends to be a problem with this style of package (Jackson et al., 1992). Also once the product is placed under retail lights the quality life of the product is only 2–4 days (depending on product), which is still about double what the retailer gets now with the traditional PVC wrap (Belcer, 2006). High oxygen/tray/barrier over wrap is similar to the Tray/Lid in that it has the same requirement for head space and uses the same gas mixtures. The difference with this package is that it uses a non-barrier polystyrene tray similar to what is used by retailers today and it is completely over wrapped giving it a more in-store look. The same advantages and disadvantages exist with this option as with the first (Belcer, 2006).
Gas barrier packaging materials available on the market have some drawbacks in terms of their cost, water-sensitivity, opacity and mechanical resistance. For instance, some packaging materials which have polar groups in the chemical structure like –OH (EVOH and PVOH) or –CO (PA) are markedly affected by relative humidity surrounding the packaging material. Therefore, new efficient barrier solutions have been developed in recent years in terms of 1) new barrier polymers, 2) thin and transparent vacuum deposited coatings, 3) blends of barrier and standard polymers, 4) nanocomposites, and 5) organic barrier coatings or adhesives (Lange and Wyser, 2003).
The use of multilayered film including a barrier layer might not be desirable with respect to recycling issues, but a substantial gauge is needed when the PE or PP (polypropylene) film is used alone. Theoretically, approximately 2 cm PE is required to achieve the same O_2

barrier as EVOH at a gauge of 0.4 μm, which prohibits it as an eco-friendly solution (Cerny, 1991).

MATERIALS AND METHODS

In this work, examined materials were: monomaterials (PE, PA and PET), commonly used materials for meat and meat products packaging (PA/PE, PET/PE, PA/EVOH/PE) and highly barrier foils (PVC//PE–EVOH-PE and PET//PE-EVOH-PE).
Physical, mechanical, structural and barrier properties were determined:

- Thickness, determined using micrometer with sensitivity of 0.001 mm. Five thickness measurements were carried out on each film, from which an average was obtained
- Tensile strength (MPa) and elongation at break (%), determined at device on the Instron Universal Testing Instrument Model No 4301, according to ASTM standard method D882-01
- Structure of polymers, using Nicolet iS10 FT-IR spectrometer in Attenuated Total Reflectance (ATR) technique, from 4000 to 500 cm^{-1} with 16 collected scans for each record
- Permeability of gases, using gas chromatography method Lyssy, according to DIN 53380

Descriptive statistical analyses for calculating the means and the standard error of the mean were performed using StatSoft Statistica 10.0. All obtained results were expressed as the mean ± standard deviation (SD). All graphs were performed using MicroSoft Excel software (MicroSoft Office 2003).

RESULTS AND DISCUSSION

The results showed that monomaterials pass their good properties on multilayer materials. **Material thicknesses** are shown in Figure 1.

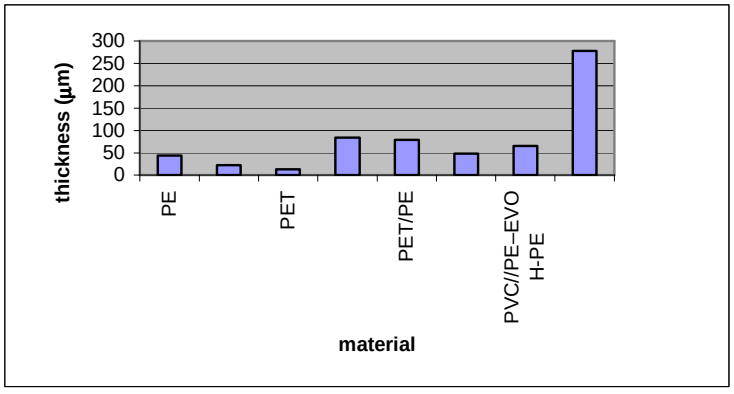

Figure 1. Thickness (μm)

Mechanical characteristics are presented in Table 1 as value of tensile strenght (N/15mm) and elongation at break (%). Results presented in Table 1 show that tensile strenght of sample PET//PE-EVOH-PE has the highest value which is in accordance with highest value of thickness of the PET//PE-EVOH-PE sample.

Table 1. Tensile strength [N/15mm] and elongation at break [%]

Material	Tensile strength [N/15mm]		Elongation at break [%]	
	Longitudinal	Transfersal	Longitudinal	Transfersal
PA	16.3±1.32	16.1±1.77	110.6±8.41	15.5±1.29
PE	13.4±1.61	12.3±1.72	240.7±31.29	710.2±113.63
PET	25.1±1.58	24.9±1.77	37.1±2.22	22.3±1.43
PA / PE	47.0±3.15	36.3±2.82	298.8±22.71	389.2±31.14
PET / PE	40.0±3.6	43.6±4.80	89.4±10.72	50.4±5.55
PA / EVOH / PE	45.7±2.24	48.67±2.68	84.42±4.89	57.72±2.95
PVC//PE–EVOH-PE	65.8±4.2	66.3±3.1	112.84±8.80	115.95±8.57
PET//PE-EVOH-PE	98.4±5.4	102.7±7.9	49.72±7.30	21.47±6.11

Results are given as mean ± standard deviation (n = 5)

Using modern techniques of Attenuated Total Reflection Infrared Spectroscopy with prior chemical layer separation **structural properties,** of individual layers of material which form part of film used for packaging meat and meat products, are presented. Figure 2 presents the following spectra (a) PA, (b) PE, (c) PET, (d) PVC.

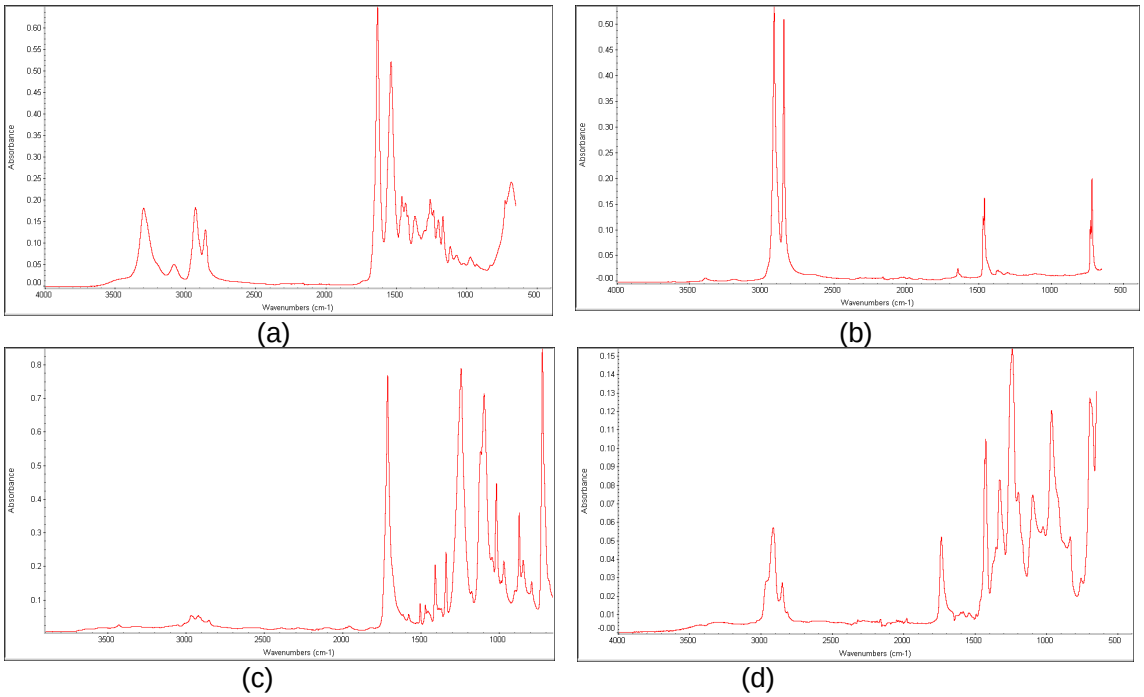

Figure 2. Spectra in different samples

Among the physical properties of packaging materials, the control of gas permeability is very important for maintaining the quality of packaged meat products. Gas permeability can be reduced by combining the base materials with other gas barrier materials through laminating, coating, blending or metalizing. The quantity of gas transmission through a packaging material depends on various factors such as the type, area, thickness and gas permeability of the film, differences in the partial pressure on both sides of the film, and storage temperature and relative humidity (Lee, et al., 2008).

Significant differences in barrier properties were obtained (Table 2). Among monomaterials the highest air permeability showed PE foil.The lowest permeability value was recorded in case of multi-layer foil type PA/EVOH/PE obtained by co-extrusion process, in which the measured value of air permeability was 4.5 ml/m^{2}24h. Obtained results point out that multi-layer materials, that contain barrier materials (PA, EVOH) and are produced by co-extrusion process, show best barrier properties.

Table 2. Gas permeability [ml/m^{2}24h]

Material	Gas permeability [ml/m^{2}24h]			
	CO_2	O_2	N_2	Air
PA	242.7±7.28	53.1±3.24	19.6±0.65	26.7±0.4
PE	7193.8±187.04	3477.2±156.47	1056.9±43.33	1570.2± 25.12
PET	760.12±31.16	248.3±6.95	251.7±4.53	250.8±15.30
PA / PE	196.9±3.74	46.9±3.89	16.7±0.70	23.1±0.62
PET / PE	21.4±0.98	120.3±6.62	115.7±4.05	118.5±2.01
PA / EVOH / PE	56.0±3.47	21.1±4.01	0	4.5±0.14
PVC//PE–EVOH-PE	23.8±1.93	15.5±0.64	0	3.6±0.12
PET//PE-EVOH-PE	23.85±1.4	16±0.57	26.85±1.46	26.65±1.62

Results are given as mean ± standard deviation (n = 3)

CONCLUSION

In the modern food chain system, it is hardly conceivable to distribute foodstuffs without packaging. Traditionally, food packaging has been limited to preservation and protection of food from environmental factors including chemical, physical and biological influences up to the point of consumption. This emphasizes retarding spoilage, extending shelf-life, and preserving the quality of packaged food. Modern packaging, however, should serve not only as an efficient tool for keeping quality of foodstuffs, but also for increasing product values, promoting sales and imparting information. With consumers ageing, factors including price, safety, size of packaging and recyclability are most important, but design, convenience and utility must also be taken into account.

This study confirmed good mechanical properties by analysing tensile strength and elongation at break of tested materials. Results of gas permeability measurements indicate good barrier properties. Examined multilayered packaging materials could be successfully used on meat packaging lines intended for vacuum or modified atmosphere packaging. Improvement of technology of meat products goes towards the development of new products with improved properties for the targeted population, the introduction of new materials and new packaging requirements, with the inevitable quality management in manufacturing.

ACKNOWLEDGEMENTS

This work is part of project „Osmotic dehydration of food - energy and environmental aspects of sustainable production“, project number TR-31055, financed by Ministry of Education and Science, Republic of Serbia.

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EFFECT OF ANTIOXIDANTS ON INHIBITION OF OXIDATIVE PROCESSES AT STORAGE OF SPREADS

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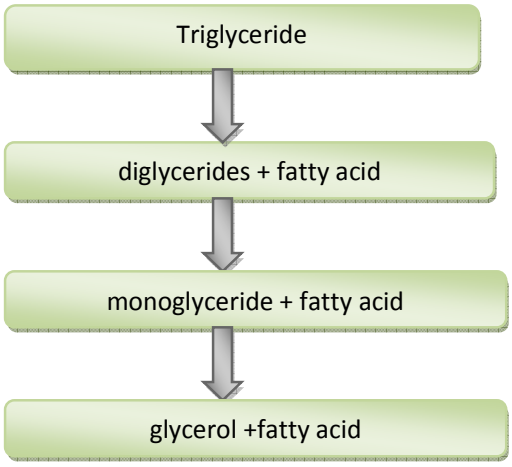
ABSTRACT: Manufacturing of butter in Ukraine has lost its profitability because there are no raw-materials. A combination of butter with oil (spread) is a new product at the Ukrainian market. Studying of the oxidation process of fats is associated with a reduced loss of fat and fat-containing products during their storage. This is important for fat, food, candy, perfume and cosmetic industries. The process of fat oxidation is a chain free radical process with branching chains. Hydrolytic and oxidative processes occur during the manufacturing and storage of fat. The final product of oxidation is malonic dialdehyde. Selection and addition of antioxidants of natural origin of the crude drug is very important. The goal of the studies was to compare the effects of various antioxidants on the stability of spreads to oxidation during storage. Phenolic compounds, natural vitamins A, E and β-carotene materials have been used as antioxidants. Samples were subjected to accelerated oxidation. There are defined organoleptic properties, as well as acid and peroxide numbers and malonic dialdehyde content in the samples. It has been shown that antioxidants exercise an inhibitory effect on the formation of peroxides. The effect of antioxidant action depends on the number of introduced additives. In most cases with increasing concentration of the additive, its stabilizing effect increases. However, there is a negative impact on the organoleptic properties of the spreads. The spreads with antioxidants have high biological and nutritional value.
Key words: *spread, antioxidant, storage, inhibition of oxidative processes.*

INTRODUCTION

Because of a shortage of raw materials in Ukraine, butter manufacture has lost its profitability. Production became not encouraging to the manufacturers (Викторова, 2007). Therefore, a new product - spreads – has appeared in the Ukrainian market. To manufacture the spreads, substitutes of dairy fat on a vegetable basis are used. The fatty acid composition of butter is regulated with the help of substitutes. But in the course of storage of the spreads, oxidative processes can lead to damaging of the product and occurrence of undesirable smacks, such as cardboard, metal, olive, grease, fish and others.

During the storage of fats, especially in adverse conditions, there are changes, which resulted in a number of chemical combinations with an unpleasant taste and odor. Fat damage can occur both under the influence of enzymes, and under the influence of air oxygen. Action of enzymes is accelerated by humidity and temperature increase, light, salts of metals of copper, iron, lead, and zinc. One can distinguish hydrolytic and oxidizing decays of fat. The kind of damage depends on the preservation of fat and conditions of its storage (Горбатова, 2003).

Hydrolysis is a process of disintegration of fat to glycerine and fatty acids. Hydrolysis of triglycerides passes the following three stages:



These stages proceed consistently, but with different speeds.
Fat hydrolysis is caused, mainly, by the enzyme of lipase. Apart from that at high humidity and storage temperature, hydrolysis occurs as a result of the influence of oxygen from air and light on the fat. Fat hydrolysis is characterized by the accumulation of fatty acids. Hydrolytic disintegration of fat results in the accumulation of high-molecular fatty acids which have no taste and smell, so they do not change the organoleptic product indicators. Occurrence of flying low-molecular fatty acids such as butyric, caproic, caprylic which possesses unpleasant smell, sharply worsens organoleptic properties of oil (Горбатова, 2003).
Oxidative damage of dairy fat proceeds at low temperatures in the presence of oxygen from air and light. Thus, there is a deep disintegration of fat which leads to formation of peroxides, aldehydes, ketones, alcoholic acids and other compounds possessing unpleasant smell. Thus, fat oxidation is connected with the formation of extraneous undesirable smacks of the product owing to the fact that the product gets various defects of smell (prorancid, silage, etc.). Polyunsaturated fatty acids are exposed to oxidation first of all.
In the initial stage of oxidation, the essential role is played by free radicals, which appear in fat under the influence of energy (light, thermal), metals, oxygen, etc.
Stability of fats one understands as their ability to remain high quality for a long time. Therefore, stability of fats defines factors that limit their damage of the chemical and biochemical origin. Speed of enzymatic and chemical processes depends on the chemical composition of fat, temperature of storage, moisture content, etc. Oxidation process is influenced by some chemical substances which either accelerate it (prooxidizers), or slow it down (antioxidants).
Antioxidants are a specific group of chemicals of different chemical composition. They possess ability to eliminate free radicals and to slow down oxidation-reduction processes.
The aim of this work was to study the influence of selected antioxidants such as vitamins A, E, carotinoids, phenolic compounds on the oxidative processes during the storage of spreads.
In view of this, the following problems have been defined:
- Selection of a way of the accelerated oxidation of fats;
- Influence of hydrolysis and oxidation processes of a spread with various antioxidants on following indicators: acid number, peroxide value and a malonic dialdehyde formation.

MATERIAL AND METHODS

For carrying out experimental studies, we prepared the spread with a mass fraction of fat 73 ± 0.5 % from cream and a substitute of dairy fat. Reference and experimental samples with antioxidants were tested for organoleptic, physical and chemical indicators. The prepared

spread samples did not differ in their physical and chemical indicators, which corresponded to the requirements of the operating standard documentation of Ukraine for the spreads.
To set up the accelerated oxidation experiment, several ways were considered:
1. At temperature 100 ºC and constant blow of air through the fat during 8–10 hours before achieving 10 mm ½ O_2;
2. At temperature 100 ºC without air purge;
3. At room temperature and absence of direct sun rays within a month;
4. At an irradiation infra-red and ultra-violet beams within 4 hours.
The first and second ways are more suitable for liquid fats, which do not contain moisture, but for the spread they cannot be used. As the spread contains up to 25 % of moisture, treatment at such a high temperature would cause a division of a product into water and fatty fractions, and also moisture evaporation that leads to chemical compound change of the spread. The third and fourth ways are suitable for research of oxidizing processes of spreads, therefore they can be used in the further work.
Spreads with antioxidants were kept at room temperature in standard conditions: thickness of the layer was 4 - 5 mm, area of the contact with air was 2.8 cm^2/g, and duration of storage in a glass case at the North side of the room under the scattered light was 20 days.
Concentration of an antioxidants was chosen not to exceed 40 % of a daily requirement for the person.
The acid number characterizes the presence of free fatty acids in a fat. It is expressed as a mass of KOH in the milligrammes necessary for neutralisation of free fatty acids, in 1 g of a fat. The method is based on titration of free fatty acids by KOH solution (concentration of KOH 0,1 mol/dm^3) with the presence of phenolphthalein as an indicator (Горбатова, 2003).
Peroxide number name is a measurement of the quantity of peroxides in fat. Definition of this indicator is based on interaction of peroxides of fat with sated water solution of KJ and iodine determination with a solution of $Na_2S_2O_3$ (concentration of $Na_2S_2O_3$ 0.1 mol/dm^3) in the presence of starch (Горбатова, 2003). The product with a peroxide number no more than 0.03 % J_2 is considered fresh.
As an end product of lipid peroxidation, malonic dialdehyde is a measurement of the efficiency of an antioxidants. During incubation in a boiling water bath in acidic medium, malonic dialdehyde reacts with 2-thiobarbituric acid, forming a coloured complex with an absorption maximum at 535 nanometers (Андреева, 1988).

RESULTS AND DISCUSSION

Addition of antioxidants in the spread essentially did not influence the organoleptic indicators of the final product. A notable difference was made by the addition of β – carotene at the concentration of 0.8 % and above, which caused a weak taste and aroma of β – carotene to be felt, and the colour became a more intensive orange color, which does not correspond to standard organoleptic indicators of a ready product.
In fresh spread samples the peroxide number was determined to be 0.028 % J_2, which corresponds to norm and indicates the freshness of the product. During the course of storage at room temperature in the presence of light and absence of direct sun rays, an increase of peroxide number values is observed. This showed that the occurrence of free radical chain reactions which are known to happen - at the first stages of formation of peroxides and hydro peroxides. Higher peroxide number of the sample without an antioxidant (control) indicates a faster damage of the product over the storage period of four weeks at room temperature. After a week of storage peroxide number of the control sample increased from 0.028 to 0.058 % J_2 and the spread still remained fresh.

Table 1. Dynamics of peroxide number changes (% J_2) of the control sample and samples of spread with antioxidants during the course of storage

Antioxidant, mass fraction	Duration of storage, week			
	1	2	3	4
control	0.058 ± 0.003	0.094 ± 0.0025	0.150 ± 0.0039	0.270 ± 0.0051
β -carotene, %				
0.2	0.042 ± 0.0021	0.069 ± 0.0031	0.098 ± 0.0032	0.170 ± 0.0041
0.4	0.036 ± 0.0015	0.061 ± 0.0025	0.092 ± 0.0029	0.140 ± 0.0039
0.6	0.032 ± 0.0014	0.037 ± 0.0016	0.061 ± 0.0025	0.097 ± 0.0032
0.8	0.030 ± 0.0035	0.033 ± 0.0017	0.056 ± 0.0021	0.086 ± 0.0037
1.0	0.029 ± 0.0010	0.032 ± 0.0015	0.051 ± 0.0020	0.074 ± 0.0031
vitamin E, %				
0.1	0.058 ± 0.0029	0.094 ± 0.0036	0.150 ± 0.0041	0.270 ± 0.0050
0.2	0.041 ± 0.0020	0.060 ± 0.0026	0.097 ± 0.0031	0.150 ± 0.0039
0.3	0.034 ± 0.0017	0.059 ± 0.0026	0.091 ± 0.0033	0.110 ± 0.0034
0.4	0.031 ± 0.0014	0.035 ± 0.0016	0.069 ± 0.0031	0.084 ± 0.0032
0.5	0.029 ± 0.0010	0.032 ± 0.0014	0.054 ± 0.0022	0.076 ± 0.0034
vitamin A, %				
0.07	0.042 ± 0.0021	0.069 ± 0.0031	0.098 ± 0.0035	0.170 ± 0.0044
0.09	0.036 ± 0.0016	0.061 ± 0.0027	0.092 ± 0.0031	0.140 ± 0.0041
0.11	0.032 ± 0.0016	0.037 ± 0.0014	0.061 ± 0.0026	0.097 ± 0.0036
0.13	0.030 ± 0.0013	0.033 ± 0.0015	0.056 ± 0.0024	0.086 ± 0.0035
0.15	0.029 ± 0.0012	0.032 ± 0.0016	0.051 ± 0.0021	0.074 ± 0.0034
extract of tea, %				
2	0.042 ± 0.0020	0.069 ± 0.0030	0.098 ± 0.0023	0.170 ± 0.0043
4	0.036 ± 0.0017	0.061 ± 0.0024	0.092 ± 0.0021	0.140 ± 0.0039
6	0.032 ± 0.0015	0.037 ± 0.0014	0.061 ± 0.0027	0.097 ± 0.0022
8	0.030 ± 0.0012	0.033 ± 0.0015	0.056 ± 0.0026	0.086 ± 0.0037
10	0.029 ± 0.0010	0.032 ± 0.0014	0.051 ± 0.0019	0.074 ± 0.0033
extract of coffee, %				
2	0.042 ± 0.0019	0.069 ± 0.0031	0.098 ± 0.0020	0.170 ± 0.0042
4	0.036 ± 0.0017	0.061 ± 0.0021	0.092 ± 0.0023	0.140 ± 0.0039
6	0.032 ± 0.0015	0.037 ± 0.0014	0.061 ± 0.0025	0.097 ± 0.0033
8	0.030 ± 0.0012	0.033 ± 0.0014	0.056 ± 0.0027	0.086 ± 0.0036
10	0.029 ± 0.0012	0.032 ± 0.0015	0.051 ± 0.0020	0.074 ± 0.0032

Results are given as mean ± standard deviation (n = 3)

In pre-production models oxidation process was slowed down depending on the quantity of added antioxidant. In spreads which contained a small amount of antioxidants, peroxide value almost did not differ from the control. With increasing of content of biologically active compounds the oxidation process significantly inhibited and in these samples it almost doesn't grow during a week. After two weeks, peroxide value of the control sample increased to 0.1 % J_2. With a such value of the peroxide number, a product has doubtful freshness. Also, organoleptic indicators changed, there was a foreign smell that indicated the beginning of disintegration of peroxides.

The best antioxidant protection was achieved with β - carotene at 1 % level of addition. Throughout the four weeks of storage, peroxide value of this sample fluctuated from 0.028 to 0.07 % J_2. Samples with peroxide value from 0.06 to 0.1 % J_2 are considered as products of doubtful freshness. Despite the best antioxidant activity, addition of 1 % β - carotene significantly changed the colour of the product to brilliant orange, which did not correspond to the natural colour of the product.

Acid number characterizes the process of enzymatic or non enzymatic hydrolysis of fat. Disintegration of fat with formation of fatty acids can promote the process of lipid oxidation as free fatty acids (not connected to triglycerides) are oxidised first of all. For this reason acid number was determined in all pre-production models. Acid number of each of fresh spreads was between 1.01 and 1.3 mg KOH.

Despite the increase in acid number which proved the accumulation of free fatty acids, changes of organoleptic indicators of the products was not observed during the storage.

After three weeks of storage, further increase in acid number to 3.25 mg KOH was observed in all samples including the control. Thus, fat hydrolysis occurred during the storage of samples of the spread at ambient temperature. Process goes almost with identical speed, both in the control sample and in the samples with tested antioxidants. The reason is that the role of antioxidants is that they inhibit the oxidation by breaking free radical chain reactions, but they don't influence the hydrolysis of atsilglitserids.
Malondialdehyde in the control sample and the samples spread with biologically active substances were determined once a week for four weeks. Pink colour didn't appear in the samples, so it can be concluded that the decay of peroxide compounds, even in the control sample did not reach the final products, one of which is malondialdehyde.

CONCLUSIONS

The conducted study has shown that introduction of antioxidants (vitamins A, E, carotinoids, polyphenols) in the spreads brakes the process of the accelerated oxidation at ambient temperature. It was established that over the course of the accelerated oxidation fat hydrolysis occurred, and that acid number of the control and pre-production models changed almost equally. Throughout the four weeks of studies, malonic dialdehyde appeared neither in the control sample nor in the pre-production models with added antioxidants.
It is clear that carotinoids, catechols, tocopherols and other compounds, which are a part of selected antioxidants, actively react with peroxide radicals, eliminating them from the oxidation chain reaction. The offered antioxidants as substances are capable of inhibiting the process of transformation of free-radical chains during oxidation. These antioxidants can be recommended for the manufacturing of the spreads intended for long-term storage, which also possess the high biological and food value.

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CHARACTERIZATION OF POLY(D,L-LACTIDE)/SILICA NANOCOMPOSITES FOR FOOD PACKAGING APPLICATION

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ABSTRACT: Currently PLA is used in food packaging application only for short shelf-life products, but designing the materials with exact properties provides many opportunities for PLA/nanocomposites for various food packaging applications. Properties of PLA can be improved by incorporation of nanoparticles into polymer matrix. Because of the small particle size and extremely high surface area, the incorporation of nanoparticles into a polymer matrix creates interphases and changes the intermolecular interaction of the matrix. As a result, by incorporation of only small amounts (less than 10 wt %) of nanofillers, a polymer nanocomposite can exhibit markedly improved physical and mechanical properties. The goal of this work was to investigate the potentials of PLA /silica nano composites for food packaging application. Neat PLA and PLA with 0.2, 0.5, 1, 2, 3 and 5 wt% of nanosilica were prepared by solution casting method in chloroform. Several procedures were used to experimentally characterize the PLA/nanocomposite samples. The size of the silica nanoparticles was measured by Zetananosizer. Differential scanning calorimeter (DSC) was used to measure the glass transition temperature (Tg) of the polymer nanocomposite samples. Thermogravimetric analysis was performed for investigation of thermal stability of all samples in inert atmosphere from 25℃ to 600℃ .
Key words: *polylactic acid (PLA), silica , nanoparticles, food packaging*

INTRODUCTION

Polylactic acid (PLA) is recyclable and compostable polymer, with high transparency, good processability and water solubility resistance. The application of pure PLA polymer for food packaging was not possible, because of the poor mechanical properties, barrier and oxidative properties that are not suitable for this kind of application. Biopolymers have increased interest because of their positive environmental impact. They are produced from renewable sources and are biodegradable, so as a result their use have less negative effect compared to conventional petroleum based polymers. Biopolymers are used in variety of applications, like therapeutic aids, medicines, coatings, food products and packaging materials (Petterson and Oksman 2006). Poly (lactic acid) PLA is biodegradable aliphatic polyester derived from 100% renewable resources (corn starch or sugar beet). Popularity of PLA has large increasement because its mechanical strength and easy processability compared to other biopolymers. It has good physical properties for diverse applications including food packaging (Zhang et.al., 2008). It can be processed by injection molding, film extrusion, blow molding, thermoforming, fiber spinning and film forming (Rasal et.al., 2010). Also it requires less energy to produce than petroleum-based polymers (25-55% less) (Rasal et.al., 2010). However PLA in most applications need some modification to enhance thermal stability, mechanical and barrier properties which is possible by filling PLA with nanoparticles (Pluta, 2004). Inorganic fillers as dispersed phases in nanocomposites are nanosized, with at least one dimension in nanometer range (1-100nm). Nanofillers can create very large interfaces with polymer matrix because of their small particle size and extremely high surface area. Incorporation of only small amount of nanofillers (less than10%) can result in improvements of material performances (Wen et.al., 2011). Nanofillers are classified according to their morphology: layered, acicular or spherical (Wen et.al., 2011). Most studies concerning PLA nanocomposites are in PLA/layered nanoparticles. Only a few studies are

published with spherical (silica) nanoparticles. Never the less, spherical (silica) nanoparticles are, because of their great natural abundance, low cost and high thermal resistance and surface functionality very suitable for various applications (Wen et.al., 2011).
Food packaging has the role to protect packaged goods from outside influence and damage, to contain food and to provide that fresh undamaged food gets to consumers (Marsh and Bugusu, 2007). Currently PLA is used in food packaging application only for short shelf-life products, but designing the materials with exact properties provides many opportunities for PLA/nanocomposites for various food packaging applications (Zhang et.al., 2008). The future design of materials gives the opportunity not only to be environmental friendly, but also to satisfy various applications. PLA/nanoreinforced materials are one of the most promising materials for the future times. Scientist and industry stakeholders have already identified potential uses of nanotechnology in virtually every segment of the food industry including food packaging (Duncan, 2011). However, understanding of the stability of PLA under realistic food packaging conditions is essential to match the shelf-life of the foods with the shelf life of the packaging material (Ahmed et.al.2010). The goal of this work was to investigate the potentials of PLA /silica nanocomposites for food packaging application.

MATERIAL AND METHODS

The PLA used in this study was provided from Symbra Technology, Netherlands. The molecular weights of PLA were determined at temperature 25℃ by gel permeation chromatography (GPC) using Agilent 1100 Series system with refractive index, RID 1200, and diode array 1200 detectors. The column ZORBAX PSM 300 was used. The rate flow of the eluent tetrahydrofuran was 1 cm^3min^{-1}. The average molar masses, *Mn*, *Mw* and polydispersity index *Q* were determined using software Agilent ChemStation. Poly(styrene) standards were used for calibration curve. Parameters of the neat PLA from (GPC) are: Mn=60520; weight-average molecular weight Mw=160780, and polydispersity of Q=2.6. The nanosilica (AEROSIL®R812) was supplied by Evonik (Hanau, Germany), and was hydrophobic, with specific area of 260±30m^2g^{-1}, average particle size 7nm. They were used as received without any pre-treatment.

Sample preparation

Neat PLA and PLA films with 0.2, 0.5, 1, 2, 3 and 5 wt % of nanosilica were prepared by solution casting method in chloroform. Silica was measured, added in chloroform and stirred in ultrasonic bath for 10min. PLA was added to solvent and stirred with magnetic bar for four hours on 40℃. After completely dissolved in chloro form samples were poured into glass Petri dishes (10 cm diameter) and vacuum dried. Chloroform was evaporated and the films were peeled of the Petri dishes.

Characterization

Zetananosizer ZS (Malvern instruments) was used to measure the average size of the nanoparticles in chloroform. The measurements were performed in quartz cuvettes.
The dispersion of the silica nanoparticles in the PLA matrix and the silica-matrix interface were studied by scanning electron microscopy (SEM), performed in a JEOL JSM-6460LV microscope operated at 25 kV. Samples were cryogenically fractured and the fracture surface was sputter-coated with very thin gold layer, in a Bal-Tec SCD-005 instrument, before scanning.
Tensile strength was measured on instron dynamometer Toyoseiki AT-L-118A equipment.
Thermal properties of the samples were measured using a DSC model Q20 (TA Instruments, USA). Aluminum pans containing 3-5 mg of polymers were hermetically sealed. The non-isothermal curve of obtained material was scanned from 20 to 200 ℃. The heating rate was 10 ℃ min^{-1}.
Thermogravimetric analysis was performed on the TG analysis device 701 LECO (Leco Corp.,USA). Analyses was performed in defined temperature range from 25 to 600℃,

samples were subjected to thermogravimetric analysis (TGA) in an inert atmosphere of nitrogen with heating rate 10℃/min.

RESULTS AND DISCUSSION

Through sample preparation silica nanoparticles were dispersed using ultrasonic bath, and good dispersion was detected on Zetananosizer (Figure 1). Samples of PLA/silica nanocomposites with 1% silica content were recorded on scanning electron microscope (SEM). Visible nanoparticles on SEM (Figure 2) have size of approximately 50nm which confirms the good dispersion of silica into polymer matrix and confirmation that there was not any agglomeration in PLA/silica samples

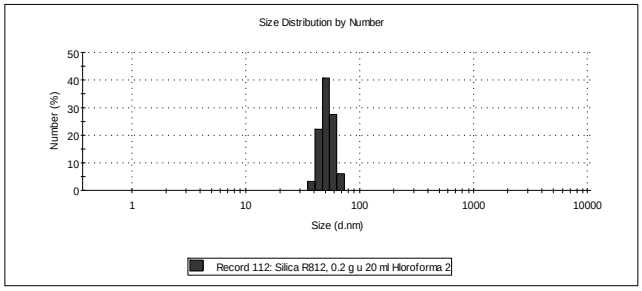

Figure 1. Number size distribution for PLA with 1% silica content

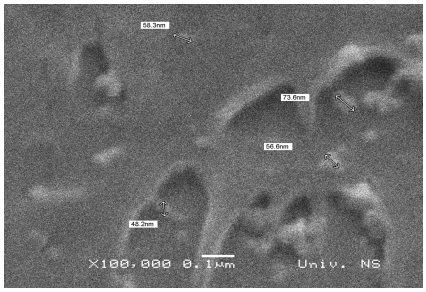

Figure 2. SEM of samples PLA with 1% silica content

Mechanical properties of prepared nanocomposite samples are improved especially for very small concentration of nanosilica particles. The most significant increasing of tensile strength was recorded for the sample with 0.2% silica (Figure 3), as well an improvement for samples with 0.5% and 1%. Decreasing of tensile strength start as the content of silica increased. For 5% content of silica value of tensile strength is below then neat PLA. This behaviour for higher content of nanoslilca is probably because of agglomerate formation, which causes decrease in mechanical properties.

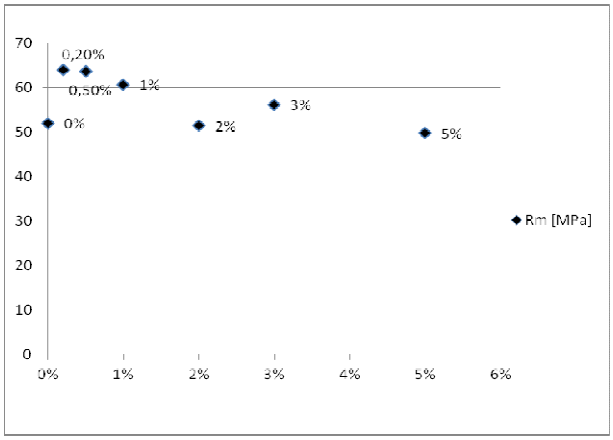

Figure 3. Tensile strength (Rm) dependant of filler content for PLA with different percentage (0,0.2,0.5,1,2,3 and 5%) of silica

The DSC thermograms indicate that the glass transition temperature (Tg) decreased as the filler content in the polymer matrix increased (Figure 4). This result may be attributed to the polymer chains moving due to the presence of the filler between the polymer chains. It has been shown that segmental dynamics of the polymer chains could reduce the Tg (Ristić at.al. 2012). The explanation of this phenomenon can result in improvement of mechanical

behaviour of the hybrid materials which was confirmed in the present study by the composites' measured tensile strength.

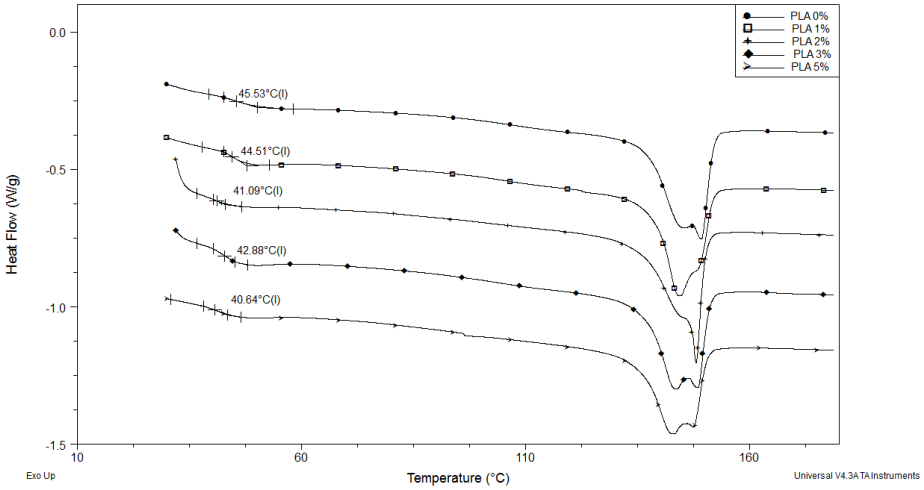

Figure 4. DSC thermogram PLA with different percentage of silica (0,1,2,3 and 5%) nanoparticle defined in figure as PLA (0,1,2,3 and 5%)

Thermogravimetric analysis shows that there are no significant changes in thermal stability (Figure 5). Same figure shows that at the higher temperature there are differences in degradation model due to different percentage of nanosilica.

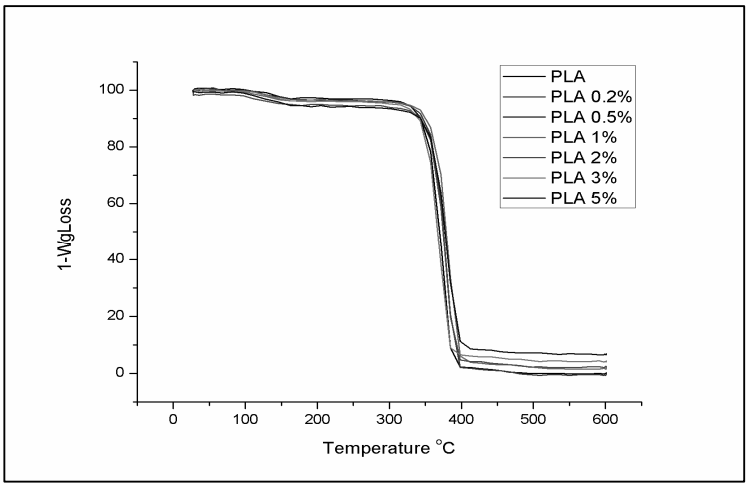

Figure 5. TGA curves for different percentage PLA with different percentage (0,0.2,0.5,1,2,3 and 5%) of silica nanoparticles

CONCLUSIONS

The simple method for PLA/silica sample preparation gave good results concerning distribution of nano particles. Spherical nano particles had very good dispersion in solvent, as well as in prepared films. From the results it can be concluded that the role of spherical nanoparticles is crucial for improvements of mechanical properties of PLA matrix. Incorporation of nanoscale silica particles into PLA matrix shows the improvement in tensile strength for adition of 0.2% concentration of nanosilica. Thermal stability of all samples did

not show any significant improvement. However, glass transition temperature decreased as the percentage of silica increased, due to polymer chain movement..

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III46001 “Development and utilization of novel and traditional technologies in production of competitive food products with added value for national and global market - CREATING WEALTH FROM THE WEALTH OF SERBIA”, financed by the Ministry of Education and Science, Republic of Serbia.

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SELECTION OF OPTIMAL THERMAL PROCESS PARAMETERS FOR THE PRODUCTION OF SEA BUCKTHORN BEVERAGES WITH HIGH ANTIOXIDANT ACTIVITY AND LONG SHELF-LIFE

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ABSTRACT: Sea buckthorn (*Hippophae rhamnoides L.*) is a unique medicinal and aromatic plant and belongs to the family of *Elaeagnaceae*. Sea buckthorn juice, derived from the sea buckthorn berries, provides a nutritious beverage, high in suspended solids and rich in vitamin C and carotenes. In view of its highly perishable nature, sea buckthorn juice has to be processed in order to extend its shelf-life. The objective of this study was to evaluate the effect of mild heat treatment on microbial behavior and the shelf-life extension of sea buckthorn beverages during storage at temperatures from 5 to 20 ℃. Two different beverages were produced by mixing sea buckthorn juice with concentrated fruit juices from orange or pomegranate. These beverages were evaluated through storage time. Thermal treatments were applied at 60, 70 and 80 ℃ for 1, 3 and 5 min. Total aerobic microflora and yeasts and moulds were enumerated through the whole storage period at appropriate time intervals. Total antioxidant activity was measured for produced samples. Sensory evaluation was performed before microbial analysis of all samples. All thermal treatments delayed the recovery and the growth of all studied microorganisms during storage. All the obtained results were kinetically described. The effect of temperature on the deterioration rates was studied. The shelf-life of all beverages was determined based either on the growth of microorganisms or on the reduction of antioxidant activity, depending on the storage temperature.
The optimum process conditions were chosen based on the shelf-life extension which was estimated according to the higher antioxidant activity and the simultaneous lower microorganisms' growth rates.
Key words: *Sea buckthorn, beverage, extended shelf-life, antioxidant activity*

INTRODUCTION

Nowadays, there is a trend in consumption of fruits, vegetables and other derived plant products due to their beneficial effects on human health mainly attributed to the presence of bioactive molecules such as phenolic compounds (Crozier, Jaganath, & Clifford, 2009). These phenolic compounds including flavonoids, phenolic acids and tannins are a major group of phytochemicals which exhibit strong antioxidant (Pietta, 2000) and antibacterial activities (Mayer et al., 2008; Saleem et al., 2010). *Hippophaë rhamnoides L*., commonly known as sea buckthorn, is an Eurasian nitrogen-fixing actinomycetes plant species, producing yellow-orange berries at the end of summer (Rousi, 1971), from which beverages, jams, candies and cosmetics are manufactured. Sea buckthorn has recently gained in interest for its nutritional and medicinal values (Guliyev et al., 2004). The juice contains an oil phase trapped within the suspended solids. The juice of sea buckthorn is well known for its antioxidant properties, attributed to hydrophilic and lipophilic compounds including ascorbic acid, flavonoids, proanthocyanidins and carotenoids (Fan et al., 2007; Gao et al., 2000; Michel et al., 2011; Rösch et al., 2003). Antimicrobial activities have also been reported for sea buckthorn berries and juice (Puupponen-Pimiä et al., 2001). In the literature there are limited cited papers describing the effect of process conditions and storage time and conditions on the shelf-life of sea buckthorn beverages.

The objective of our work was to refine the formulas for the production of two sea buckthorn beverages, to select the optimum thermal process conditions for pasteurization and to study their shelf-life.

MATERIAL AND METHODS

In our study, two different beverages were produced by mixing the sea buckthorn juice with concentrated fruit juices from Navel var. orange and pomegranate. In general, for both beverages, the 50% was orange and pomegranate reconstituted juice, respectively, 10% was sea buckthorn juice and the rest was water. These beverages were evaluated for their stability through storage time. The quality indices that were measured are described below:

Microbiological analysis
Total aerobic viable count, yeasts and moulds were determined. Two replicates of at least three appropriate dilutions were enumerated. All plates were examined visually for typical colony types.

Color measurement
Quantification of the color change was based on measurement of CIELab values with a CR-200 Minolta Chromatometer (Minolta Co., Chuo-Ku, Osaka, Japan) with an 8 mm measuring area. At predetermined times of isothermal storage, according to the design, measurements were conducted and values of ΔC and ΔE were determined.

Sensory analysis
The sensorial attributes of sea buckthorn beverages were evaluated by a trained sensory panel of 5 people. Panelists were asked to score appearance, color, freshness, odor, taste and overall impression of beverages using a 1 to 9 descriptive hedonic scale.

Antioxidant activity
Antioxidant activity of the samples was expressed through the determination of the effective concentration (EC_{50}). The EC_{50} value expresses the amount of sea buckthorn beverages necessary to decrease the absorbance of DPPH by 50% (Antolovich et al., 2002). The value can be determined graphically by plotting the absorbance against the used extract concentration.

Storage conditions
Sea buckthorn beverages were stored at controlled isothermal conditions of 5 and 15 ℃ and at air temperature (approximately 25 ℃). Sampling was carried out at appropriate time intervals and kinetic analysis of microbial growth, texture analysis, color measurement, pH and sensory analysis were carried out.

Thermal treatment
The effect of temperature treatment on the sea buckthorn beverages stability was examined. The beverages were pasteurized (thermally treated) in thin-walled aluminum spiral placed in water-baths at temperatures in the range of 60-80 °C for 1 to 5 min. The treatment was continuous, using a peristaltic pump to push the liquid into the spiral. After thermal processing, the spiral was immersed into water bath of 15 ℃ for fast cooling. Aseptic packaging of the beverages followed the cooling into glass bottles of 150 ml each. During thermal treatment temperature was monitored in order to assure isothermal conditions.

RESULTS AND DISCUSSION

Thermal treatments were applied at 60, 70 and 80 ºC for 1, 3 and 5 min. Total aerobic microflora and yeasts and moulds were enumerated before and after processing. Total

antioxidant activity was measured for treated and untreated samples. The results indicated that thermal treatment significantly affected the initial microbial load of the beverages and their total antioxidant activity. Higher temperatures combined with longer treatment resulted in decreased colonies forming units and total antioxidant activity (results for sea buckthorn beverage containing pomegranate are shown in Table 1).

Selection of thermal treatment conditions

All thermal treatments resulted in reduced microbial load and reduced total antioxidant activity. The selection of process conditions for the shelf-life experiments was based on the higher antioxidant activity, the not detectable microbial load and the higher score of sensory evaluation. Treatment at 80 ℃ for 1 min resulted in higher antioxidant activity and sensory scoring with decreased number of viable cells (Table 1).

Table 1. Effect of thermal processing temperature and time on the quality indices of sea buckthorn and pomegranate beverage

Treatment temperature (ºC)	Treatment time (min)	Total microflora (logCFU/ml)	Yeasts and moulds (logCFU/ml)	EC_{50} (ml of beverage)	Sensory evaluation
Control	Control	4.75	3.52	0.247	9
60	1	3.54	3.17	0.574	9
	3	2.41	2.28	0.625	9
	5	<2	<2	1.058	8
70	1	2.34	2.11	0.847	9
	3	<2	<2	1.147	8
	5	<2	<2	1.358	8
80	1	<2	<2	0.941	8
	3	<2	<2	1.847	7
	5	<2	<2	2.752	6

Shelf-life experiments

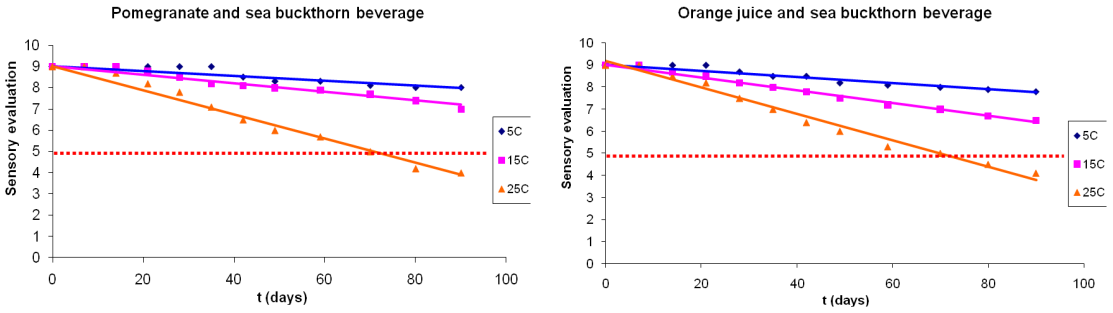
Thermal treatment at 80℃ for 1 min delayed the recovery and the growth of all studied microorganisms during storage for more than 3 months. Microorganisms were under the detection limit for all beverages during the whole storage period.

Color was significantly affected for beverages stored at 25 ℃ (Table 2), while for samples stored at 5 and 15 ℃ the color degradation was slower (Table 2). The same trend was observed for sensory evaluation of the beverages. For those stored at 5 ℃ the sensory degradation was significantly low, while for those stored at 25℃ a bitter taste was observed after approximately 60 days of storage (Figure 1).

Table 2. Effect of storage time on the ΔE color parameter of sea buckthorn beverages

Storage time (days)	Pomegranate and sea buckthorn beverage			Orange juice and sea buckthorn beverage		
	5 ℃	15 ℃	25 ℃	5 ℃	15 ℃	25 ℃
0	30.6	30.6	30.6	47.1	47.1	47.1
7	31.8	32.7	35.7	47.7	48.5	51.7
14	32.2	34.3	39.3	44.1	49.2	52.7
21	33.5	35.9	42.9	45.7	49.9	57.9
28	38.4	40.2	46.2	47.2	50.7	54.8
35	37.4	41.2	48.8	48.4	51.7	57.7
42	39.6	44.8	51.7	50.2	52.4	58.1
49	42.1	46.1	54.2	51.5	57.5	59.4
59	43.5	48.7	57.1	52.7	54.8	71.7
70	44.7	49.8	60.2	55.4	57.7	77.4
80	48.5	52.4	62.3	57.7	58.7	75.7
90	49.7	55.1	64.8	59.8	71.2	77.4

The organoleptic deterioration rate constants for each storage temperature for both beverages were estimated (Table 3). Increase of storage temperature resulted in significantly higher deterioration rates as expected.



(a) **(b)**

Figure 1. Effect of storage time on the sensory evaluation of (a) pomegranate and sea buckthorn beverages and (b) orange juice and sea buckthorn beverage. The red dot line represents the acceptance/non-acceptance limit for the products

Table 3. Organoleptic deterioration rate constants of sea buckthorn beverages at different storage temperature

Storage temperature (℃)	Sea buckthorn + orange juice beverage organoleptic deterioration rate constant ($days^{-1}$)	Sea buckthorn + pomegranate juice beverage organoleptic deterioration rate constant ($days^{-1}$)
5	0.0111	0.0136
15	0.0201	0.0286
25	0.0565	0.0597

Shelf-life determination

The quality degradation and the shelf-life estimation of the developed beverages can be correlated to the development of off-odour in the products and by the color alteration. Taking into consideration all the received results from our study, the shelf-life can be determined based on color alteration (ΔE-value equal to 60) or the sensory evaluation (scoring equal to 5). The shelf-life of the sea buckthorn beverage with pomegranate is estimated as 325, 180 and 70 days (Table 4), after storage at 5, 15 and 25 ºC, respectively. For the orange juice and sea buckthorn beverage, the corresponding shelf-life is estimated as 285, 130 and 70 days (Table 4), respectively for the same storage temperatures. Comparing the received results with corresponding results for untreated samples stored at 5 ºC (shelf-life estimated as 7 and 5 days, respectively), a 40-fold increase is achieved when pasteurizing at 80 ℃ for 1 min.

Table 4. Shelf-life (days) determination of sea buckthorn beverages at different storage temperatures

Storage temperature (℃)	Sea buckthorn + orange juice beverage	Sea buckthorn + pomegranate juice beverage
5	285	325
15	130	180
25	70	70

CONCLUSIONS

The development of two commercially viable sea buckthorn beverages with fresh-like characteristics and extended shelf-life was achieved. Optimum time and temperature conditions (80 ℃ for 1 min) for pasteurization wer e selected, based on microorganisms and enzymes inactivation while maintaining the nutrients and sensorial quality. The quality degradation and the shelf-life estimation of the developed beverages can be correlated to the the color and sensory degradation. In general, the shelf-life is estimated as 180 and 130 days for storage at 15 ºC, for sea buckthorn beverage with pomegranate and orange juice, respectively.

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SURVEY OF OVERALL MIGRATION FROM DIFFERENT KIND OF METAL PACKAGING LACQUERS

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ABSTRACT: Metal cans as an economic and useful packaging usually have a polymeric coating, and it is possible that different compounds can transfer from this coating to foodstuff. If this transferring includes known and unknown compounds, it will be known as Overall migration. In this study overall migration caused by metal can polymeric coatings in food stimulants, is investigated. Investigated metal can lacquer coatings, were from golden phenolic epoxy kind, beige phenolic epoxy kind, and white Andric epoxy kind, and 10% ethanol and 3% acetic acid was used as stimulant for overall migration testing. The Overall migration was calculated by determination the lose weight of lacquers after storage at mentioned conditions. Result showed that overall migration ranges obtained from golden phenolic epoxy, beige phenolic epoxy, and white Andric epoxy liquors are around 2-4.2 mg/dm^2, 1.65-4.9 mg/dm^2, 2-4.2 mg/dm^2 , respectively. Results show that the amounts of overall are adopted with (European Union) EU legislation that identifies as overall migration limit (OML).
Keywords: *Can, Lacquers, Overall migration*

INTRODUCTION

Polymeric coatings or lacquers prevent reaction between metal and food. Epoxy phenolic lacquers with gold and beige appearance and epoxy anhydride lacquers with white appearance are more useful coatings in metal cans (Forrest, 2005; Nehring, 2007; CFR/175300, 2007). Migration in food from packaging is transferring parts or compounds from packaging to foodstuff and it caused by contact or reaction between packaging and foodstuffs (Schaefer, 2004; Ruyon et al., 2002; Lund et al., 2000). Specific migration from packaging to foodstuff follows mass transferring so it is necessary to know about mass lows and diffusion phenomenon. Migration is in accordance to Fick's low (i.e. migration has direct relation with concentration of present compounds in packaging and temperature) (Piringer 1994; Schaefer, 2004). Migration in food packaging science is studied from two point of view: overall migration and specific migration (Piringer,1994 ; Uematsu et al., 2004). Overall migration includes monomers and oligomers and intermediate compounds with molecular weight below 1000 Dalton and can be determined by gravimetric method (Commission Directive, 2002; CEN/ EN 1186-1,2002; CEN/TS 14235, 2002).Overall migration test in foodstuffs in order to its heterogeneous nature and possible interfere, is impossible. Therefore simulants are used to determination real value of overall migration (Piringer et al., 2000; Piringer et al., 2008). In order to determine overall migration importance allowed limits of overall migration have been determined in different countries by internal and international authorized organizations. This values in accordance with EU standard must be 10 mg/dm^2 of surface or 60 mg/kg in weight (Commission Directive, 2002; CEN/ EN 1186-1, 2002; CEN/TS 14235, 2002). In This study overall migration from polymeric coatings of metal can coatings which they are used in packaging industrial in Iran has been investigated. Then the results have been compared with standards.

MATERIALS AND METHODS

Materials

Metal cans were coated with golden epoxy phenolic, beige epoxy phenolic, and white epoxy anhydride lacquers, obtained from four main metal can producer factories; they were coded as A, B, C, D, Also the used chemical (Ethanol 96%, Acetic acid, Distilled water) were purchased from Merck Co., Germany.

Equipment

Analytical balance with 0.1 mg accuracy, Oven capable to 110^0C, Quartz or stainless steel containers, containers

Method

Simulant preparing

Acetic acid 3%

Weight 30ml acetic acid and then pure it into a 1000ml ground neck flask and poured with distilled water.

Ethylic ethanol 10%

Pure 104ml ethylic ethanol (96%) was poured into 1000ml ground neck flask with distilled water.

Preparing samples

Empty cans with golden epoxy phenolic, beige epoxy phenolic, and white epoxy anhydride coatings were placed in 110ºC oven, maintain for 30 min until they reached stable weight and then, placed in a desiccator. Then samples had been transferred to desiccators, and after they had been cooled, they were weighted by an analytical balance. Then cans were filled with acetic acid (3%) and ethanol (10%), and then they were sealed.

Applying thermal and time conditions

Filled cans had conditioned similar to food production process. For foods that are sterilized during production, filled cans with simulant were placed in 121^0C (sterilization temperature) for 1h. Then cans had placed in 40^0C oven for 10days (The recommended overall migration testing).So the real condition of heating that they were being used by industry can be obtained.

Determination of overall migration

After exposure can samples to thermal and time treatment, simulants had completely discharged from cans and purred in special quartz, stainless still containers that had previously reached constant weight, and evaporated on hot plate. After evaporating simulant, special containers had placed in 110^0C oven for 30 min and after cooling in desiccators, had weighted again. Obtained residues from evaporating represent overall migration and the results were expressed in mg/dm^2 of surface of metal can in contact with foodstuff.

In the same time migration value were determined in blank simulants to determine the residue of simulant after evaporation (Commission Directive, 2002; CEN/ EN 1186-1, 2002; CEN/TS 14235, 2002).

Calculations

Overall migration had calculated by this formula:

$$M = \frac{[(m_a - m_b) - m_d] * 1000}{S}$$

M; the overall migration to acetic acid 3% and ethanol 10% in mg/dm^2 in surface

m_a; initial weight of sample before exposing to simulant in gram

m_b; sample weight after exposure to simulant in gram

m_d; blank weight in gram

S; surface of sample, which had exposed to simulant in dm^2

Statistical analysis

Experiments on each of samples were performed at four replicates. Two analyses were taken from the test samples at each specific time interval. Statistical analysis (Mean values and standard division were calculated at each time interval, so were analyzed by SPSS ver. 11.5 (SPSS Inc. Michigan Avenue., Chicago, USA) and Minitab ver 11.12 (Minitab Inc., State College , USA)

RESULT AND DISCUSSION

1- Overall migration average value results for white epoxy anhydride and golden epoxy phenolic in A, B factories in acetic acid 3% and ethanol 10% simulants

Results obtained from determination overall migration value in white epoxy anhydride and golden epoxy phenolic in A, B factories in acetic acid 3% and ethanol 10% simulants are in table 1.

Table1. Result of overall migration for epoxy anhydride and golden epoxy phenolic lacquers

Food Simulant+ Factory / Lacquer	Overall migration (Mg/dm^2)			
	Ethanol 10%		Acetic 3%	
	B Factory	A Factory	B Factory	A Factory
Golden Epoxy Phenolic	3.8882	2.738	4.254	2
White Epoxy Anhydride	0.65	3.2606	2.7042	1.4462

Overall migration results for golden epoxy phenolic lacquer in A factory in ethanol 10% was higher than in acetic acid 3% and the results of overall migration for same compounds in B factory in acetic acid 3% was higher than in ethanol 10% simulant. Also the results of overall migration for white epoxy anhydride lacquer in A factory in ethanol 10% was higher than amount in acetic acid 3% and the results of same migrated compounds in B factory in acetic acid 3% was higher than in ethanol 10%. Moreover amount of migrated white epoxy anhydride lacquer in A factory in ethanol 10% was higher than in acetic acid 3% simulant but in contrast the mentioned amount of migrated compounds in B factory in acetic acid 3% was higher than in ethanol 10%.

Results shows that particle migration value from white epoxy anhydride lacquer in ethanol 10% and in acetic acid 3% have a significant difference($P<0.05$), whereas migration value in both ethanol 10% simulant and acetic acid 3% simulants have not a significant difference. Studying on overall migration in golden epoxy phenolic lacquer and white epoxy anhydride lacquer in acetic acid 3% simulant and ethanol 10% simulant that reported as mg/dm^2 in two factories A and B showed that there is a significant difference ($P<0.01$). Statistically analysing of A and B, also relation between lacquers and factories showed that there is a significant difference in amount of migration ($P<0.05$). Finally, considering both three parameter; factory, lacquer kind, and simulant we can report that there are significant differences in overall migration.

According to overall migration analyse, lacquer kind had highest effect on the amount of overall migration while factory and simulant kind had second and third grade, respectively.

2- Overall migration average value results for white epoxy anhydride in A - B- C- D factories in acetic acid 3% and ethanol 10% simulant

Overall migration value results for white epoxy anhydride in A - B- C- D factories in acetic acid 3% and ethanol 10% simulant are in table 2.

Table 2. Result of overall migration for epoxy anhydride lacquer in different factories

Lacquer + Factory Food Simulant	Overall migration (Mg/dm^2)			
	White Epoxy Anhydride			
	A	B	C	D
Acetic 3%	1.4462	2.7042	4.9196	1.541
Ethanol 10%	3.2606	0.65	2.266	0.873

Amount of Overall migration for white epoxy anhydride lacquer in A, B, C and D factories in acetic acid 3% and ethanol 10% simulant, showed this amount in acetic acid 3% simulant in C factory was the highest, and in A factory was the lowest. Also in ethanol 10% simulant in a factory was highest and in B factory was lowest. This fact is depended on some factors such as Tinplate sheets kind, lacquer formulation kind, quality of lacquer fill it expire date, lacquer storage condition, etc. Significant differences.($P<0.01$) were observed in overall migration values between acetic acid 3% and ethanol 10% simulants.

3- Overall migration average value results for white epoxy anhydride and beige epoxy phenolic in C factories in acetic acid 3% and ethanol 10% simulants

Overall migration value results for white epoxy anhydride and beige epoxy phenolic in C factories in acetic acid 3% and ethanol 10% simulants are included in table 3.

Table 3. Result of overall migration for epoxy anhydride and beige epoxy phenolic lacquers

Lacquer + Factory Food Simulant	Overall migration (Mg/dm^2)	
	Beige Epoxy Phenolic	White Epoxy Anhydride
Acetic 3%	1.6526	4.9196
Ethanol 10%	3.3454	2.266

Overall migration value in white lacquer in 3% simulant and ethanol 10% simulant in C factory was different to overall migration value in beige lacquer. This may caused by difference in stability or instability of these coatings to each one of two simulants and reaction of lacquers in contact with acid and ethanol. In addition, present difference between lacquers, can be related to formulation of each one of lacquers (in a view of resin base, and ingredients, etc,) and sheet kind and lacquer coating thickness on sheet surface etc. In addition, adhesion ability and plasticity of coating film and quality of consistence coating can also be effective on difference in migration value.

Overall migration in different coatings and acid and ethanol simulants in different factories

Summary, overall migration results were obtained in studying different metal container internal coatings in contact with food simulants (acetic acid 3% and ethanol 10%) has shown in table 4.

Table 4. Result of overall migration in different lacquers with simulants in different factories

Factory	Lacquer	Simulants	migration (Mg/dm^2)
A	White Epoxy Anhydride	Acetic 3%	1.4462
A	White Epoxy Anhydride	Ethanol 10%	3.2606
A	Golden Epoxy Phenolic	Acetic 3%	2.00
A	Golden Epoxy Phenolic	Ethanol 10%	2.738
B	White Epoxy Anhydride	Acetic 3%	2.2704
B	White Epoxy Anhydride	Ethanol 10%	0.6278
B	Golden Epoxy Phenolic	Acetic 3%	4.425
B	Golden Epoxy Phenolic	Ethanol 10%	3.8882
C	White Epoxy Anhydride	Acetic 3%	4.9196
C	White Epoxy Anhydride	Ethanol 10%	2.266
C	Beige Epoxy Phenolic	Acetic 3%	1.6526
C	Beige Epoxy Phenolic	Ethanol 10%	3.3454
D	White Epoxy Anhydride	Acetic 3%	1.541
D	White Epoxy Anhydride	Ethanol 10%	0.8732

CONCLUSION

Results obtained in this study can give some useful and practicable data about contaminants migration situation in internal produced metal cans. According to EU committee (EC) legislations, coatings must do not transfer their ingredients to foodstuff in values higher than 10 mg/dm^2. Studies showed that all examined samples have overall migration were adopted by specific migration limit, in accordance to EN14235. This showed that produced lacquers have desirable properties, and metal can producers followed safety and technical principals and legislations considering sheet kind and can applications in accordance to package foodstuffs and responsible person's controls was effective.

ACKNOWLEDGEMENTS

This study was supported by Food and Drug Control Labs, Ministry of Health of Iran. The Authors are grateful staff of Packaging and Toxicology Labs, Food and Drug Control Labs for their helpful cooperative during this project.

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MODERN TRENDS OF FOOD PACKAGING

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ABSTRACT: Packaging has an active role in processing, preservation and retaining the quality of the food. The growing consumers demands for the food that are ready to eat, fresh-tasting, nutrient and vitamin rich and minimally-processed and preserved are a major challenges for the current food industry. In order to realize these demands more significant role is gained to the packaging techniques and materials. Development in packaging is focused primarily towards the better protection of packaged food products, greater reliability, increased functionality, better environmental attributes of packaging, as well as in the development of new materials and packaging technology. In this paper the method of modified (protective) atmosphere packaging, biodegradable packaging as well as active and intelligent packaging are discussed. In addition the advantages of nanoscale materials for application in food packaging is also reviewed.
Key words*: MAP, biodegradable packaging, active and intelligent packaging, nanoscale materials*

TRENDS IN FOOD PACKAGING

Due to increasing consumers demands for the food that are mildly preserved, fresh, tasty with a prolonged shelf-life, the food packaging industry has rapidly developed to meet and satisfied these expectations. The global trends such as increased industrial processing of food, greater importation and exportation of food products, and less time for preparation of fresh foods are the driving force for the food and beverage packaging industry to investigate advanced packaging solutions (Lazić et al. 2008).

Modified Atmosphere Packaging (MAP) is a technique used for prolonging the shelf-life of fresh or minimally processed foods (Church, 1994). Protective atmosphere enables that packaged food products maintain their visual, textural and nutritional properties. Modifying of the gas atmosphere in food packaging could be achieved by removing gas (vacuum packaging) or by replacement the air in the packaging by introducing the desired gas composition. For achieving the desired atmosphere in the packaging air could be mechanically replaced by the desired gas or gas mixture, the desired atmosphere in the package could be achieved passively, by metabolism (respiration) of packaged product and the desired gas atmosphere can be implemented actively, by adding absorbers or emitters of certain gases in the packaging. Today, MAP has become the dominant packaging method for some products, like fresh pasta, chilled meats, snacks and dried food, cooked meats and seafood, bakery goods like bread, muffins, croissants, pizza etc. In addition, fresh fruits and vegetables, particularly prepared salads have also been packed in MAP. Figure 2. shows binding of oxygen and releasing of carbon dioxide and water vapor in packaged fruit, and their transport through packaging. The composition of the gas which will be used for the MAP depends on the packaged product (Lazić et al, 2007). In order to assess and calculate the gases and water vapor permeability of polymer films, depending on the speed of "breathing" of packaged products and the desired concentrations of gases, corresponding software has been developed (Jovanović and Džunuzović, 2011).
In order to improve the mechanical, thermal and gas barrier properties of polymer films used in MAP, the introduction of nanocomposite material could be of great importance (Brody,

2003). Nanocomposite materials might dramatically increased the barrier properties comparing with conventional ones, such as lower levels of transmission of oxygen. For example, the transmission of oxygen in Nylon-6 nanocomposite was 4 times lower than in the conventional nylon-6 (Brody, 2003).

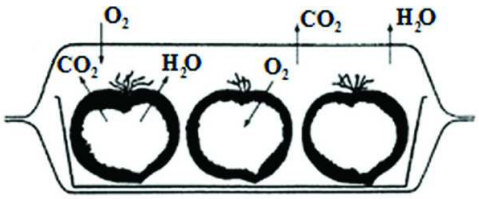

Figure 1. Formation, bonding and diffusion of O2, CO2 and
Water vapor during packaging of fresh fruits and vegetables in a partially permeable atmosphere
(Jovanović and Džunuzović, 2011)

BIODEGRADABLE MATERIAL FOR PACKAGING

There is an increasing demand for identifying biodegradable packaging materials and finding innovative methods to make plastic degradable (Lazić and Gvozdenović, 2007). Biomaterials are polymers produced from renewable resources (polysaccharides, lipids, proteins etc). Biodegradation is the process by which carbon-containing chemical compounds are decomposed in the presence of enzymes secreted by living organisms (Lazić et al., 2008). In addition, edible packaging refers to the use of edible films, coatings, pouches, bags and other containers as a means of ensuring the safe delivery of food product to the consumer in a sound condition (Mc Hugh et al., 1994). These films can also act as carrier of antioxidant, flavor and antimicrobials and can improve mechanical integrity of food products (Seydim and Sarikus, 2006).

ACTIVE AND INTELLIGENT FOOD PACKAGING

Traditionally, food packages have been defined as passive barriers to delay the adverse effect of the environment on the packed product. However, the modern trends include the development of packaging materials that interact with the environment and food, playing an active role in their preservation.
Active packaging changes the conditions of the packaged food in order to extend shelf life or to improve the safety or sensory properties, while maintaining the quality of packaged food (Brody et al., 2001; Lopez-Rubio et al., 2004). For example, oxygen scavengers remove oxygen from food packages, thereby prevents the growth of aerobe microbes and preserving the desired flavor and odor of foods. Carbon dioxide emitters suppress the growth of anaerobes in products such as meat, poultry, and cheese (Lopez-Rubio et al., 2004). Moisture-control agents suppress water activity, serving to remove fluids from products, prevent condensation from fresh produce, and prevent lipid oxidation (Kerry et al, 2006). Also, maintaining humidity in packages is accomplished by humidity controllers. Other tools used as active packaging components include antimicrobials and ethylene absorbers, emitters of preservatives such as ethanol, sorbate or benzoate, absorbers of light of specific wavelength, which adversely affects the packaged product as well as flavor releasing/absorbing systems (Tab.1.)

Table 1. Examples of active packaging applications for use within the food industry (Kerry et al. 2006)

Absorbing/scavenging properties	Oxygen, carbon dioxide, moisture, ethylene, flavours, taints, UV light
Releasing/emitting properties	Ethanol, carbon dioxide, antioxidants, preservatives, sulphur dioxide, flavours, pesticides
Removing properties	Catalysing food component removal: lactose, cholesterol
Temperature control	Insulating materials, self-heating and self-cooling packaging, microwave susceptors and modifiers, temperature-sensitive packaging
Microbial and quality control	UV and surface-treated packaging materials

Quality and safety are two of the key requirements in the food manufacturing supply chain - from packaging, distribution and food retailing through to the consumer. The intelligent packaging is designed to monitor the quality of packed food (Brody et al., 2001; Kerry et al., 2006). Time temperature indicators (TTIs), ripeness indicators, biosensors, and radio frequency identification devices (RFID) are examples of intelligent packaging components. TTIs indicator provides an affordable and widely used solution for the monitoring of products that are sensitive to time/temperature abuse, offering an easy-to-read irreversible signal that estimates the time the threshold temperature has been exceeded.

RFID provides wireless monitoring of food packages through tags, readers, and computer systems (Wanga et al., 2006). Its use in the food industry are numerous and range from facilitating the traceability of food to improve supply chain efficiency. With an appropriate sensors in the chip the relevant information about the quality of the products, such as temperature, moisture, gas composition etc. might be permanently collected. If the predicted values entered with the program are exceeded for some reasons the alarm is activated. All collected data from the chip can be transferred to the computer and the “life path of the product” from the day of production until consumption could be reproduced.

Cheap, thin and flexible electronics, which can be applied to flexible polyester substrate, can be easily integrated in packaging and in particular packaging of polymeric materials.

It is expected that this technology will soon completely replace the traditional use of bar codes (Jovanović and Džuzunović, 2011).

NANOTECHNOLOGY IN FOOD PACKAGING

Food nanotechnology is an area of emerging interest and opens up a whole universe of new possibilities for the food industry. The science about very small materials is ready to have a major impact in food packaging materials (Sanguansri and Augustin, 2006). Promising results and applications are already being developed in the areas of food packaging and food safety.

Nanotechnology may be used to produce packages with stronger mechanical, thermal and barrier properties. These properties may be developed by using *nanocomposites* (Henriette and de Azeredo, 2009).

Nanocomposites are materials that are made up of nanoparticle components. In food packaging, montmorillonite clay is being explored as the nano-component in a variety of polymers: polyethylene, polyester, nylon, and starch. Nanocomposite plastic films block oxygen, carbon dioxide, and moisture from reaching food, so when used as packaging, the material extends the shelf life of food (Henriette and de Azeredo, 2009). Nanocomposite food packages are also light, strong, and heat resistant. In addition, research into the development of biodegradable nanocomposite packages is in progress. The use of bionanocomposites for food packaging not only protects the food and increases its shelf life but also help to manage the world’s waste problem (Henriette and de Azeredo, 2009). Edible nanomaterials could find applications in fresh fruits and vegetables, bakery products and

confectionery, where they might protect the food from moisture, lipids, gases, off-flavors and odors.
In addition, the nanomaterials that might have a great potential for use in the field of food packaging is *nanotubes*. A nanotube is a nanometer-scale wire-like structure and most frequently composed of carbon (Sozer and Kokini, 2008). It has been discovered that carbon nanotubes possess antimicrobial effects (Kang et al., 2007). Direct contact with aggregates of carbon nanotubes proves to be fatal for the cells of Escherichia coli. The hypothesis is that the cellular demages occurs when long, thin nanotubes puncture the cells of E. coli, (Kang et al., 2007).
Also, carbon nanotubes can be incorporated into polymer structures (liquids, solutions, melts, gels, amorphous and crystalline matrices) to increase their mechanical properties in terms of tensile strength and elasticity (Ruoff and Lorents, 1995). In addition, particulary important to the food sector is the possibility to obtain nanotubes by partial hydrolysis of milk protein α-lactalbumin (Figure 2). The resulting α-lactalbumin nanotubes are able to increase viscosity (due to their large surface area) and stiffness, (Graveland-Bikker and de Kruif, 2006). Moreover, α-lactalbumin nanotubes have cavities of 8 nm in diameter, which might enable the binding of food components, such as vitamins or enzymes. These cavities could also be used to encapsulate and protect nutraceuticals or to mask undesirable flavor or aroma compounds (Graveland-Bikker and de Kruif, 2006).

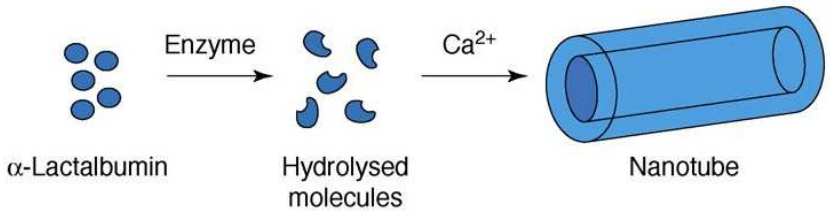

Figure 2. Partially hydrolised α-lactalbumine into nanotubes in the presence of Ca^{2+} (Sozer, N. and Kokini, 2009)

The following example of nano-sized solution with positive effect for the future of food packaging is *nanosensors*, that might be integrated with food packaging. These nanosensors can detect pathogens, toxins and chemicals in the food product. In fact, an array of thousands of nanoparticles could be designed to fluoresce in different colors in contact with food pathogens. For example, nanosensors have been developed to detect *Staphylococcus* enterotoxin B, *E. coli*, *Salmonella* spp. and *Listeria moncytogenes* (Liu et al., 2007). Nanosensors can also detect allergen proteins to prevent adverse reactions to foods such as peanuts, walnuts and gluten. Nevertheless, progression in the field of application of nanotechnology moves cautiously because the effects of nano-sized materials on humans' health are still under investigation.

CONCLUSION

Food products follow the progress in the food packaging processes and implementation of new developments in the food packaging technology. It is obvious that in the future it could be expected the greater use of MAP and CAP technology, the application of active and intelligent packaging, development and increasing application of new types of biopolymers, development and use of nanotechnology, tendency for the formation of brand and greater concern about the environmental aspects of packaging. However, regarding the implementation of nanomaterials in the food sector, there are social and ethical issues that must be well considered. Governments should consider appropriate labeling of these products and should also impose the packaging regulations that will help to increase consumer acceptability to these new materials.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education and Science, grant No. 46010.

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FOOD COLD CHAIN MANAGEMENT AND OPTIMIZATION

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ABSTRACT: Reliable information on the cold chain conditions, the main shelf life determining post-processing parameter, is an essential prerequisite for effective shelf life management of food products. Application of an optimized quality and safety assurance system for the distribution of chilled and frozen products requires continuous monitoring and control of storage conditions. FRISBEE is a Food Refrigeration Innovation for Cold Chain European project. Within FRISBEE a user friendly web-based cold chain database (http://frisbee-wp2.chemeng.ntua.gr/coldchaindb/) is being built. At all stages of the cold chain, the needs of consumer and European industry were considered, gaining a greater insight into deviations between real cold chain data and targeted specifications. Systematic data collection and processing allowed identification and evaluation of the weak links of the cold chain of chilled and frozen products. Data from all stages (production to consumption) of the cold chain were collected from researchers, industry, distributors, retailers and consumers. The developed FRISBEE cold chain database web based platform linked to appropriate shelf-life predicting tools offers the potential to effectively manage and improve cold chain weak links using appropriate shelf-life decision systems leading to an optimized handling.
Using the response of Time-Temperature Integrators (TTI), inexpensive, smart labels that show an easily measurable, time-temperature dependent change reflecting the temperature history of the food product, the integral effect of temperature can be monitored, and quantitatively translated to food safety and quality, from production to the point of consumption. SLDS (Shelf Life Decision System) and SMAS (Safety Monitoring and Assurance System) are TTI chill chain management systems that lead to an optimized handling of products in terms of quality and safety risk.
Key words: *cold chain, management, TTIs, FRISBEE*

INTRODUCTION

The main shelf life determining post-processing parameter in the chill chain of chilled and frozen food products is temperature. Practice and industrial studies have shown that temperature conditions in the distribution, handling, transport and storage of products often deviate from the recommended ones. Thus, monitoring, recording and controlling them becomes crucial for product's safety and quality as well as for shelf life predictions and expiration date labeling. Application of an optimized quality and safety assurance system for the chilled distribution of a food product requires good knowledge of the conditions of the particular cold chain. A comprehensive European data base for identification and evaluation of the weak links of the cold chain for different types of chilled and frozen products is necessary. Additionally continuous monitoring and control of storage conditions, from production to consumption would be required. Cost effective monitoring of temperature conditions of food products in the chill chain can be realized by Time Temperature integrators (TTI), inexpensive, active "smart labels" based on physicochemical, chemical or biological principles of operation and exhibiting an easily measurable response that integrates the temperature history of the product (Taoukis and Labuza, 2003). TTIs can serve as temperature monitors and tools for the optimization of stock rotation policies and chill chain management in general. Prerequisite for application of TTI is a correlation system to translate TTI response to the quality status of the food at any point of the chain. Basic structural elements of this system are validated kinetic models of TTI response and kinetics of the degradation indices of the food, such as predictive models of microbial growth. Based

on reliable models of the shelf life and the kinetics both of the product and the TTI response, the effect of temperature can be quantitatively translated to food quality, from production to the point of consumption (Taoukis, 2001; Taoukis, 2011). SLDS (Shelf Life Decision System) (Giannakourou et al., 2001) and SMAS (Safety Monitoring and Assurance System), based on the real quality and risk profile of products, use the information from the TTI response at designated points of the chill chain, ensuring that the temperature-burdened products reach consumption at acceptable quality level. SMAS system was evaluated by running a large number of chill chain scenarios using a Monte Carlo simulation approach. Experiments at chill chain conditions also demonstrated and quantified the improvement at the time of consumption in comparison to the conventional First In First Out (FIFO) approach (Koutsoumanis et al., 2005; Tsironi et al., 2008; Vaikousi et al., 2009).

MATERIAL AND METHODS

A systematic data collection for identification and evaluation of the weak links of the cold chain for different types of chilled and frozen products is necessary in order to gain quantitative insight into deviations between real cold chain data and targeted specifications. Within European FRISBEE project ("Food Refrigeration Innovations for Safety, consumers Benefit, Environmental impact and Energy optimisation along the cold chain in Europe", www.frisbee-project.eu), a web-based platform has been built for time-temperature (t-T) data collection, maximizing information retrieval with user friendliness (http://frisbee-wp2.chemeng.ntua.gr/coldchaindb/). In this platform, all t-T files were correlated to their meta-data and organized according to: Stage of the Cold Chain -Food Storage temperature range- Characterization of food-Type of food- Food product-Packaging- Country of origin. A software tool linked to the web data base was developed that allows the building of a most likely t-T distribution for selected stages of the cold chain for a specific food product, through Monte Carlo simulations on the numerous relevant t-T profiles drawn from the database, and calculates the shelf life loss of the product from stage to stage, based on shelf life kinetics.
For developing and demonstrating the TTI based management systems two TTI technologies were used. An enzymatic and a solid state photoactivatable TTI. The enzymatic TTI are based on a color change caused by a pH decrease which is the result of a controlled enzymatic hydrolysis of a lipid substrate. The Check Point® TTI type M (VITSAB, Malmo, Sweden) starts from initial green colour, becomes progressively green-yellow and reaches a final yellow color. By controlling type of enzyme substrate and concentration of the lipase the length and the temperature sensitivity of the TTI can be set. The OnVuTM TTI (BASF, Germany), is based on the inherent reproducibility of reactions in crystal phase (Patent EP 1049930 B1). Photosensitive compounds such as benzylpyridines and spyropyrans are excited and coloured by exposure to low wavelength light. This coloured state (dark blue) reverses to the initial colourless at a temperature depended rate. By controlling the type of the photochromic compound and the length of UV light exposure during activation the length and the temperature sensitivity of the TTI can be set. Kinetic modelling of response was based on measurements, at appropriate time intervals, of the response of a number of both TTI tags, isothermally stored at constant temperatures (from 0 to 15ºC). TTI color change was measured instrumentally using the Eye-one Pro (X-Rite, Michigan, USA) at D50 illumination and 2° observation angle conditions. Kinetic modelling of TTI response was based on measurements, at appropriate time intervals, of the response of 5 TTI tags, isothermally stored in high-precision low-temperature incubators at constant temperatures. The response of the TTI was modelled by defining a mathematical function that better describes the response vs time at all temperature and initial charging conditions. Model coefficients were calculated by non linear regression using SYSTAT 10.2® Software.
As example case studies, kinetic models for growth of spoilage bacteria in modified atmosphere packed (MAP) minced beef and fish fillets were used to select appropriate TTI, based on their modelled response. The aim was to evaluate the potential of applying TTI in an optimized distribution management system.

RESULTS AND DISCUSSION

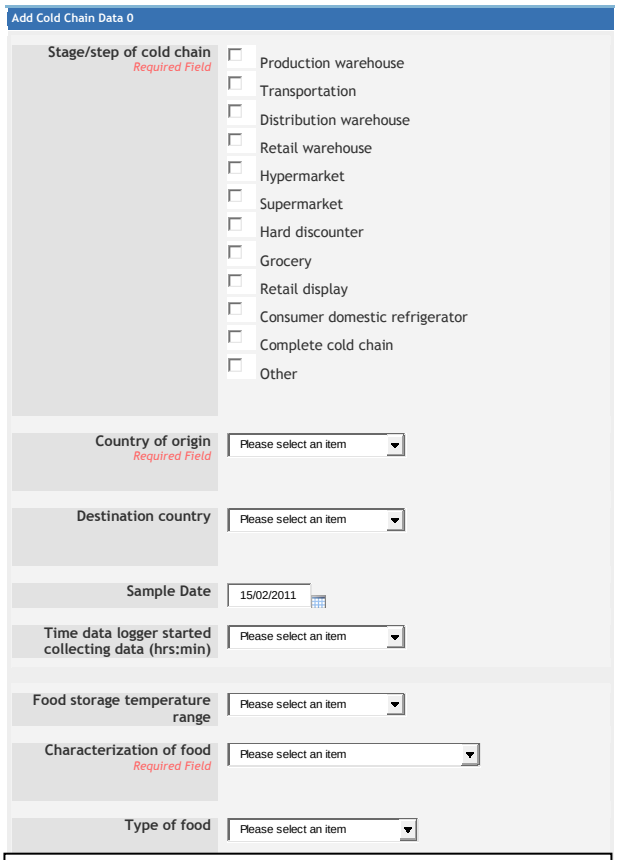

Figure 1. FRISBEE Cold Chain Data Collection

In the Data Collection platform (Figure 1), data from industry, cold chain parties (distributors, retailers) and consumer surveys, including all stages of the cold chain (from production to consumption) are continuously collected. All contributors have privileged access to this database (by login and password) and the access to the database is secured. This platform consists of a menu driven web-based software retrieving information to accompany the contributed food product time-Temperature data. The inputs are building a comprehensive and extensive database which will serve as a valuable tool to people that are involved in the Cold Chain as researchers or industrial players. All received data are further processed in such a way that the output of the web-based database includes (Figure 2):

- Actual Time-temperature profiles
- Mean, min and max value of temperature for the whole time-Temperature profile
- Effective temperature of the time-Temperature profile: To demonstrate the integrated effect of the temperature variability on product quality, the term of the effective temperature T_{eff} is introduced. T_{eff}, which is defined as the constant temperature that results in the same quality value as the variable temperature distribution over the same time period, is based on the Arrhenius model and integrates, in a single value, the effect of the variable temperature profile T(t):

$$T_{eff} = \int_0^t - \frac{E_a \cdot T_{ref}}{R \cdot T_{ref} \cdot \ln \frac{k(T(t))}{k_{T\,ref}} - E_a} \cdot dt$$

where E_a the activation energy of the quality index, T_{ref} the reference temperature, k(T(t)) the rate costant of the quality index change (e.g. growth of a microorganism) at temperature T which in turn is a function of time, k_{Tref} the rate of the quality index alteration at the reference temperature, t the time of the variable profile and R the universal gas constant.

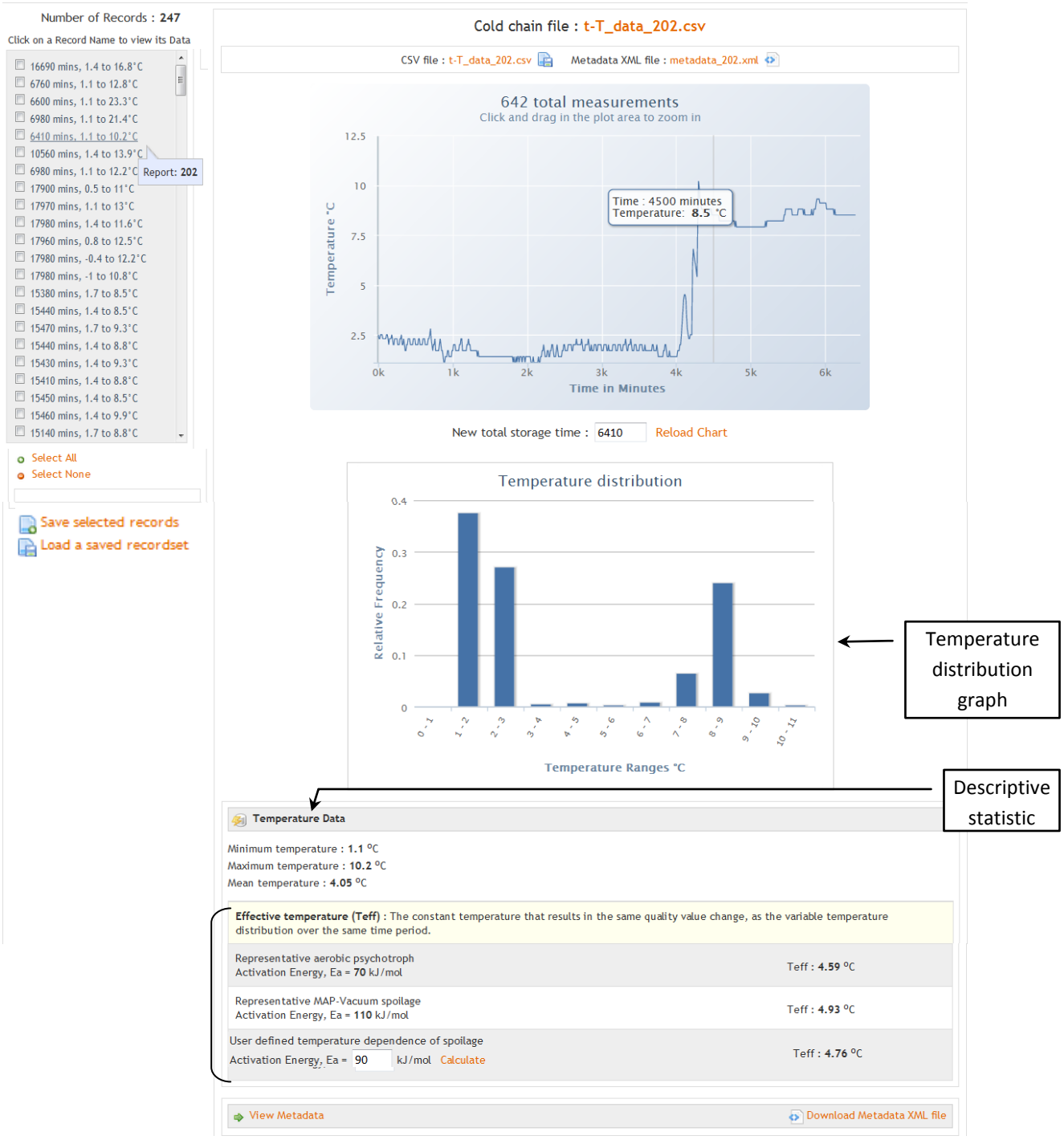

Figure 2. Example of the Cold Chain Database output

In this database, the user can build a specific sequence of cold chain stages for specific food product. The FRISBEE Cold Chain Predictor (FRISBEE_CCP), , allows the user to calculate the remaining shelf life of a specific food product at different stages of the cold chain corresponding to a specific time-temperature profile, if deterioration factor kinetic data are known (Figure 3).

Using this tool, one can efficiently manage the food cold chain and do corrective actions to predetermined stages of the cold chain for certain food products. This could result in significant increase of the remaining shelf-life at the end of each stage.

Additionally actual monitoring of storage conditions can be realized by TTIs. TTIs allow such control down to product unit level.

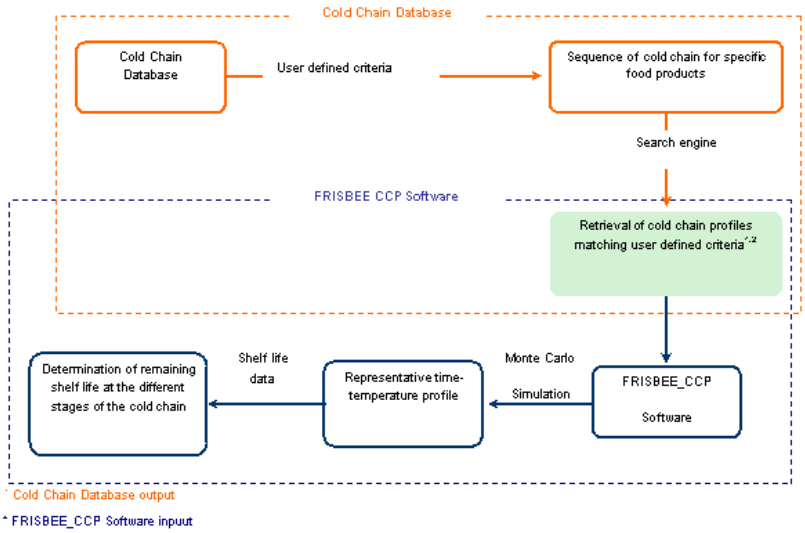

Figure 3. Architecture of the Cod Chain Database and FRISBEE_Cold Chain Predictor Software

Based on the results of the testing of the TTI, composite models that allow the calculation of their response rate, k, at any selected enzyme concentration or charging times were developed. These models allow the selection of the suitable TTI design to much the shelf life kinetics of the targeted food. For example it was calculated that charging time of 2 s for the OnVu TTI and 50U for the M-type Check Point TTI could lead to suitable TTIs for monitoring the quality of MAP minced beef during refrigerated storage (Taoukis et al., 2010). The effectiveness of several TTI based systems was evaluated by simulated chill chain experiments based on the level of dominant deterioration factor of food products at the end of their storage. For example, for the case of MAP gilthead sea bream fillets, the FIFO approach resulted in 6 out of 40 samples to have reached the spoilage level at the end of storage period. When the TTI based management was applied, only 2 of the 40 samples reached the spoilage level, significantly reducing the number of rejected products before the "time of consumption" (Figure 4) (Tsironi et al., 2011).

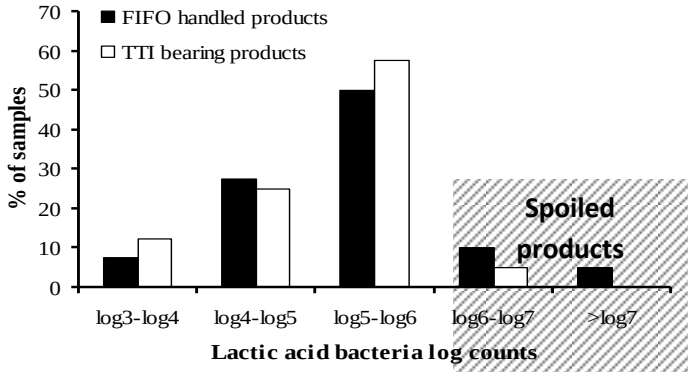

Figure 4. Distribution of the calculated lactic acid bacteria log counts in MAP (50% air-50% CO_2) gilthead seabream fillets based on FIFO and TTI based management system (Tsironi et al., 2011)

Overall, taking into consideration several respective studies for different food products conducted in our laboratory, it is concluded that the TTI based system may result in reducing the number of spoiled products. The use of TTIs at appropriate points of the chill chain (e.g. at a central distribution centre) could significantly help making decisions for the further management of products based on their temperature history and hence quality and safety status.

CONCLUSIONS

Two different approaches are proposed for the management of the cold chain of food products. In the first approach, the use of appropriate developed tools developed within FRISBEE project is proposed. The aim is the estimation of the remaining shelf-life of food products after each stage of the cold chain, based on t-T profiles inputted in a cold chain database. The contributed data of the cold chain will allow one to run simulations and distribution scenarios based on real cold chain data. Corrective actions could be applied for maximizing remaining shelf-life. The other approach is the application of TTIs which allow such control down to product unit level. The selection and use of the optimum TTIs for a particular product, with regard its visual response characteristics and temperature sensitivity, could lead to realistic control of the cold chain, reduction of waste and efficient shelf life management. If the temperature conditions of the products could be continuously monitored by TTIs, reliable estimation of the quality status and the remaining shelf life could be performed, allowing better management and optimization of the cold chain from production to the point of consumption.

ACKNOWLEDGEMENT

This review is based on results partly obtained with the financial support of the Commission of the European Communities. (RTD program “Quality of Life and Management of Living Resources”,Project N° QLK1-CT2002-02545 (http://smas.chemeng.ntua.gr), FP6 Collective Research Project COLL-CT-2005-012371 (www.freshlabel.net), FP7 Capacities Project IQ-Freshlabel (G.A. no: 243423) (www.iq-freshlabel.eu), and FP7 RTD project FRISBEE (GA no: 245288) (www.frisbee-project.eu)

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MICROBIAL POLYSACCHARIDES: BETWEEN OIL WELLS, FOOD AND DRUGS

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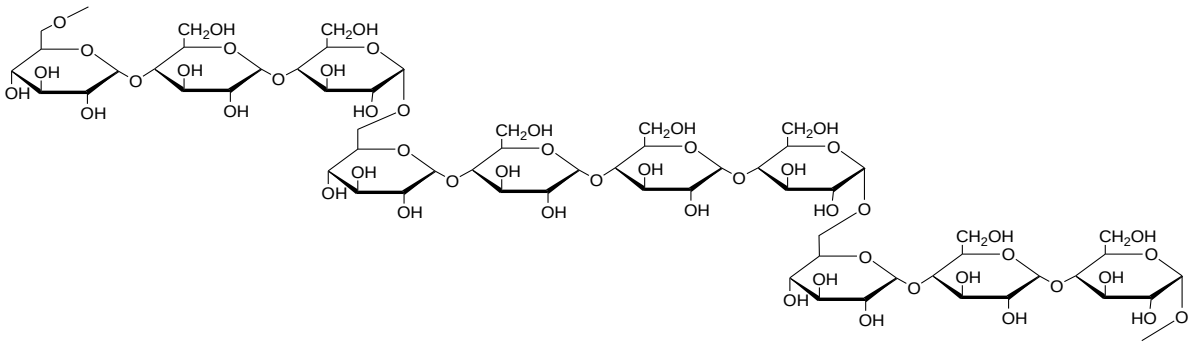
ABSTRACT: Microbial exopolysaccharides (MPSs) such as xanthan, dextran, gellan or pullulan have been commercially used, in their natural or modified state, for many years.
A large number of these natural polymer applications is a consequence of their excellent physical and chemical properties, based on their capacity to alter the basic properties of water (e.g. thickening or gelling). In addition, these polymers have related secondary functions, such as emulsification, suspension, stabilization, encapsulation, flocculation, film forming, binding and coating.
MPSs, and particularly exopolysaccharides have many other novel properties to offer, and discovery of immune modulation and bifidogenic effect of some of them should provide other applications.
This work focuses on the more recent developments in the extent of application of microbial polysaccharides in the various fields what makes these polymers promising and versatile materials in future, and also on our investigations within these natural products.
Key words*: microbial polysaccharides (MPSs), properties, material, application, industry*

BASIC KNOWLEDGE AND APPLIED ASPECTS

The ability to produce polysaccharides is widely found among different species, but despite many sources of these biopolymers, those from algae and higher plants are dominant on the world market. Polysaccharides derived from microorganisms, including bacteria, yeasts and moulds, are still not exploited enough. The main reasons for that are linked to the costs of production, because of specific substrate requirements in certain cases, bioreactors demands or obtaining aseptic conditions. Nevertheless, polysaccharide production from microorganisms has many advantages: it takes much less time compared to plants; in the case of some algae species it is more energy efficient because of use of solar energy for production; and a lot of industrial wastes and raw materials can be used as carbon sources, which is probably the greatest advantage of all (Donot et al., 2012).
MPSs are synthesized and accumulated mostly after the growth phase, and, in regard to their location in the cell, they can be divided into three main groups. Inside the cell, as carbon and energy sources are cytosolic endopolysaccharides. The second group is made of those that make up the cell wall. Polysaccharides exuded into the extracellular environment are known as exopolysaccharides (EPSs), and they appear in the form of capsules or slime. They are also involved in biofilm formation, where they have many significant roles: participation in attachment to a surface, formation and stabilization of biofilm structure, enhancement of resistance to environmental biotic and abiotic stresses and antimicrobial agents, preventing of desiccation and assumption of nutrients (Shia and Zhua, 2009).
MPSs are divided into two groups: homopolysaccharides, made up of a single type of monosaccharide (e.g. pullulan,dextran.or levan) and heteropolysaccharides, made up of several types of monosaccharide, with complex structures (e.g. xanthan or gellan). Structures of some MPSs are shown in Figure 1. In any case, they are mainly composed of



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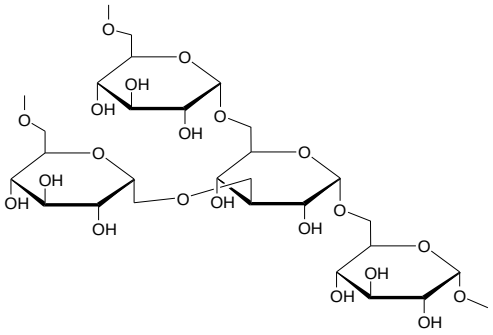

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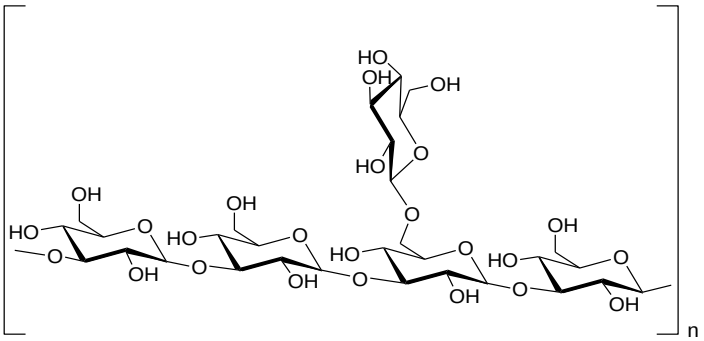

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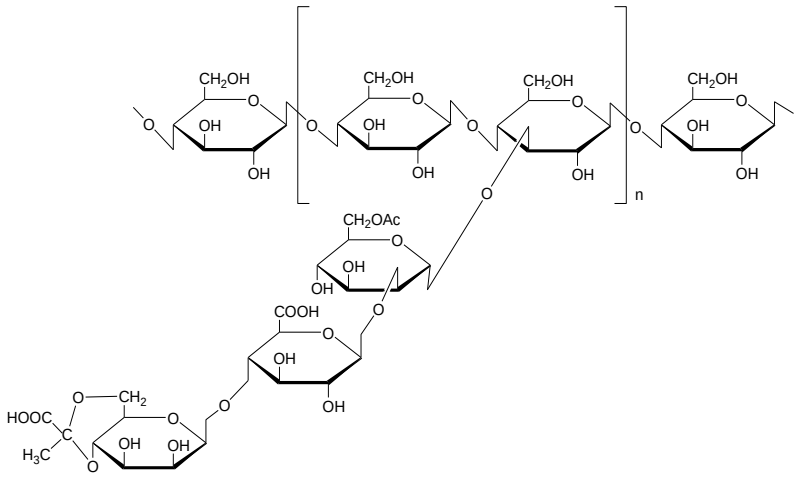

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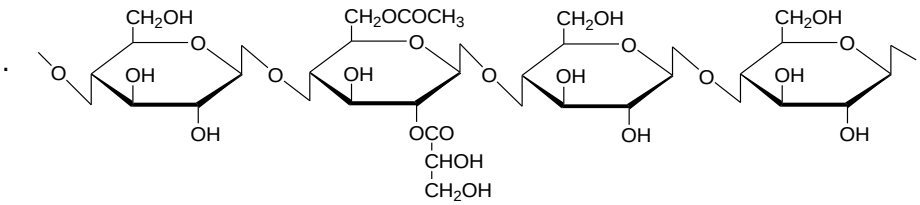

1.5

Figure 1. Structure of some widespread MPSs.
Homopolysaccharides: 1.1. Pullulan; 1.2. Dextran and 1.3. β-glucan (frequently component of cell wall-endopolysaccharide). Heteropolysaccharides: 1.4. Xantan and 1.5. Gellan.

glucose, galactose and mannose, but many other neutral, amino sugars and uronic acids are often present, too. Also they can contain some organic ester – linked substituents and pyruvate ketals, which give them anionic character and increase their lipophilicity (Freitas et al., 2011). Despite of differences in monosaccharide composition, they differ in charge, type and configuration of glycosidic linkages (which affects the rigidity of molecule), molecular weight, length and frequency of branches (from which rheological properties depends) (Duboc and Mollet, 2001).

EPSs have many various functions for microbial cells. Their role is mainly protective, against competition or extreme environmental conditions. On the other hand, their specific functional physicochemical properties are also the main reason for great interest for their application in various fields of industry – food, cosmetics, pharmaceutical, as well as in biomedicine and ecology. Despite that, only several have been industrially used since today (Freitas et al., 2011). Since they are involved in biofilm formation, they can make some serious problems (e.g. in food, process and paper industry, cooling water systems), because of causing food and water reservoirs spoiling, toxin production, pipes blockage, corrosion of the process equipment, etc. (Shia and Zhua, 2009).

One of the most important applications of microbial polysaccharides in industry is in drilling fluids for oil recovery, sequestration of toxic compounds, activating of sludge settling, suppressing of gasoline vaporization and chemical absorption of carbon dioxide. They are also used as depollution agents, and in processes of biofloculation, settling and dewatering of activated sludge (Neyens et al., 2004). Due to their texturing properties, microbial polysaccharides (such as xanthan, galactomannans and native or modified starch), independent or in combination with some other polymers, are widely applied in controlling the rheological properties and stabilizing oil-in-water emulsions (Desplanques et al., 2012).

The special rheological properties of xanthan, polysaccharide isolated from *Xanthomonas campestris* makes this polysaccharide very suitable for application in many industries, but especially in oil industry, for 'enhanced oil recovery' applications. In petroleum industry, it is used for oil drilling, fracturing, pipeline cleaning, and also in micellar – polymer flooding as a tertiary recovery operation (Palaniraj and Jayaraman, 2011). Emulsan, a lipopolysaccharide from *Acinetobacter calcoaceticus* is also able to stabilize oil-in-water emulsions, just like some other complexes made of polysaccharides, proteins and lipids (Lang, 2002).

Cyanobacterial EPSs, which are complex heteropolysaccharides, have a strong anionic character due to high content of uronic acids. Because of large number of negative charges, these polymers can effectively remove heavy metal ions from water solutions, and therefore can be used in remediation of polluted aqueous environments (De Philippis et al., 2011).

In food industry, microbial polysaccharides are mostly used as thickening, stabilizing, emulsifying, binding, structure creating and gelling agents, because of their high viscosity in aqueous media (Freitas et al., 2011). They need to have physicochemical properties that can satisfy some food processing conditions – variations in pH, temperature, ionic strength, influence of other food components etc. Many of EPSs have such properties, however, only two are allowed for use as additives in the food industry in Europe and United States: xantan and gellan (Donot et al., 2012).

Certain strains of lactic acid bacteria synthesize EPSs, which participate in production of fermented milk products: yoghurt, cheese, fermented cream, kefir, etc. They are very important for final texture (as biothickening agents they improve the reology of product – viscosity which makes them slimy and fluid, and elasticity which gives them firmness and gum-like properties), taste and stability of products (they bind water and limit syneresis). EPSs from lactic acid bacteria don't have only technological utility, but also have some health benefits on consumers. Their viscosity increases time that fermented product spends in gastrointestinal tract, which helps its colonization with probiotic bacteria. Also they can be metabolized by the colon microorganisms to short-chain fatty acids (acetate, propionate, butyrate), and those can not only provide energy to epithelial cells, but also play role in the prevention of colon cancer (Duboc and Mollet, 2001). Because of this property, some microbial polysaccharides are defined as prebiotics – "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a

limited number of bacteria in the colon, and thus improve host health" (Scantlebury Manning and Gibson, 2004).
Microbial exopolysaccharides can be shaped into micro/nanoparticles, scaffolds and hidrogels, which can be applied in biomedicine for drug delivery, encapsulation of bioactive compounds, imaging, tissue engineering and wound dressing. Some of those EPSs (xanthan, sulfated dextran, sulfated curdlan) posses some biological activity too, so their use is very important for design of such pharmaceuticals. Also, some formulations that contain fucose and oligosaccharides obtained by its hydrolysis are known as anti-cancerogenic, anti-inflammatory and anti-aging agents (Freitas et al., 2011). After adequate chemical modifications, they may serve as covalent carrier for drugs, e.g. antibiotics, facilitating their solubility in water while decreasing citotoxicity and retaining drug activity. The main advantages for use of such polymers in this kind of formulations are their biocompatibility, nontoxicity and biodegradability (Zhanga et al., 2011).
β-Glucans are structural polysaccharides of the cell wall of fungi, yeast, some bacteria and cereals. Those polysaccharides are non-digestible and can be, in some degree, fermented by intestinal microbial flora. They have the ability to stimulate immune system in the defense against some viruses, bacteria, protozoa and fungi, but also have anti-tumor activity even thou they have no direct cytotoxic effects. Therefore they are known as immunomodulators and anti-cancer agents. They participate in activation of macrofages, neutrophils, natural killer cells and lymphocytes. The main advantage of these supplements over conventional immune therapies is in possibility of oral administration, less costs and fewer side effects, but their independent use as therapeutics is still not enough (Chan et al., 2009; Murphy et al., 2010).
Bacterial cellulose, which has identical chemical composition as those from plants, has unique fibrillar nanostructure. It is used as component of high quality audio membranes, electronic paper, membranes of fuel cells and biomedical material (Weia et al., 2011). One of the most interesting uses is in treatment of chronic wounds, such as venous leg and diabetic ulcers, bedsores and burns, where this polysaccharide, in the form of film, provides necessary moist environment. It also helps in eliminating pain symptoms by isolating the nerve ending, provides good barrier against infection, and decreases healing time (Czaja et al., 2006). However, cellulose does not have antibacterial properties, but those can be obtained by impregnation with silver nanoparticles (Maneerung et al., 2008) or benzalkonium chloride (Weia et al., 2011).
To date, polysaccharides isolated from microorganisms are of great interest in the overall hydrocolloid market, even thou they are not represented enough. Research interest in its production is exponentially growing, especially because of possibilities of using low-cost substrates in their production and improving downstream processing, as well as possibility of metabolic engineering which allows controlled production of polymers with exact, fine-tuned properties. By altering conditions of some biotechnological process for obtaining microbial polysaccharides, such as nutrient media for growth of polysaccharide producing microorganism, carbon and nitrogen content, temperature, pH, aeration, stress conditions etc., polymers with various chemical composition, structure and consequently properties, can be obtained. In the next few years, significant increase in number of different products and technologies based on microbial polysaccharides can be expected.

REVIEW OF THE SOME OUR ESSENTIAL RESULTS IN THE MPSs RESEARCH

Our research group, Group of microbial chemistry, from Faculty of Chemistry, University of Belgrade and Department of Chemistry, Institute of Chemistry, Technology, and Metallurgy (IChTM), University of Belgrade, for several years is engaged in studying the microbial polysaccharides: dextran (in collaboration with the Faculty of Technology in Leskovac), pullulan (in collaboration with the Faculty of Technology in Leskovac) and β-glucan from the cell wall of baker's yeast (in cooperation with the fermentation industry Fermin, now Alltech-Serbia from Senta). These microbial polysaccharides are well known and have been commercial products.

Dextran is a glycan which can be characterized by its main structural feature, a backbone chain of α-(1→6)-glycosidic linked D-glucose units and *branch* points at *position 2*, 3 or 4. Strains of Leuconostoc *mesenteroides* are involved in *the production of this glucan*. Fractions of dextrans have application in medicine, biochemistry and biotechnology. One of the objects of our interest, in respect of this polysaccharide, was influence of process conditions on effects in biotechnological production of dextran by *Leuconostoc mesenteroides* (Lazic et al., 1993).

Pullulan is extracellular microbial polysaccharide which is produced by the yeast-like fungus *Aureobasidium pullulans*, strain CH-1 (IChTM, Collection of Microorganisms). This polysaccharide can be described as a linear α-D-glucan that consits of α-1,6-linked a regular repeating maltotriosyl units having about 7% of maltotetraosyl units randomly distributed in the polysaccharide chain (Jakovljevic et al., 2001.). *A. pullulans* CH-1 exhibited two beneficial effects, i.e., a biosorption of metal pollution and the production of pullulan (Radulovic et al., 2008). The choice of pullulan for object of our interest is based on the its properties such as non-toxicity, plasticity, lower permatibility of oxygen gas, and so on make it widely used in food packing, pharmacy, environmental protection and other industries.

In the last years β-glucans from cell wall many microorganisms attracted much attention due to their antitumor activity. Particular interest is devoted to β-glucans that isolated from cell wall of *Saccharomyces cerevisiae*. In addition to antitumor activity, these polysaccharides posses anti-inflammatory properties and manifest as nonspecific immunomodulators, as well as other, less significant and examined characteristics associated with their biological activity. Biological activity of the yeast cell wall glucan is the main reason for interest of our group for this polysaccharide (Zekovic et al., 2005). In this sense we perfected methods for isolation and purification of cell wall glucan from commercially available active dry yeast (Zlatkovic et al., 2003). By mild Pfitzner-Moffat oxidation of this glucan keto-aldehydo polymer was obtained (Zekovic et al., 2006). Bifidogenic properties of samples of β-glucans with different degrees of purity was investigated recently (Laugier et al., 2012).

The obtained results, as a result of interesting of our group for chemistry and applications of microbial polysaccharides should be a guideline for further work towards the study of biochemical properties of microbial polysaccharides, which are the subject of great interest in the world and in our laboratories.

ACKNOWLEDGEMENTS

Part of this paper is a result of the research within the Grant No. III 43004 ("Simultaneous Bioremediation and Soilification of Degraded Areas to Preserve Natural Resources of Biologically Active Substances, and Development and Production of Biomaterials and Dietetic Products"), supported by the Ministry of Education and Science of the Republic of Serbia.

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ANTIMICROBIAL ACTIVITY OF PHENOLIC EXTRACTS FROM OLIVE LEAVES AND GRAPE SKINS AND SEEDS – IMPACT OF ENCAPSULATION

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ABSTRACT: Olive leaves and grape skin with seeds produced as by-products in olive oil and wine production constitute a cheap source of plant material with certain amount of bio-active substances. Antioxidant, antimicrobial, anti-inflammatory and anticancer properties of the phenolic extracts were reported. The efficiency of extracts depends also on the ability to preserve their biological activity. Encapsulation of the extracts can improve stability, biological availability, hydrophobic/hydrophilic character and unpleasant taste for potential use in food preservation. Antimicrobial activity was first screened by broth microdilution method for minimal inhibitory concentrations (MICs) of phenolic extracts from olive leaves (*Olea europaea,* cv. "Leccino") and grape skins and seeds (*Vitis vinifera* L., cv. "Zelen") against 14 strains of *Candida, Pichia* and *Ogataea* sp., which were isolated from spoiled olive oil, and *Dekkera bruxellensis* from spoiled grape must. The kinetics of antifungal activity was followed by the yeast inhibition curves in YPD medium with phenolic extracts added in MICs. Finally, the impact of extract encapsulation into ß-cyclodextrin and liposomes on antifungal activity was tested. Phenolic extracts from olive leaves and grape skins with seeds expressed high efficiency against most isolates of spoilage yeasts. Encapsulated olive leaves extracts showed the same or better efficiency than non-encapsulated extracts in broth microdilution screening test and during yeast growth inhibition in liquid medium. The encapsulation into ß-cyclodextrin was more efficient than encapsulation into liposomes. Grape skins and seeds and olive leaves as plant waste materials have been proven to be a rich source of phenolic compounds with antimicrobial activity against spoilage yeasts.
Key words: *Minimal inhibitory concentration, plant phenolic extract, spoilage yeast, encapsulation*

INTRODUCTION

Grape skin and seeds are known to be a rich source of flavonoid and non-flavonoid phenolic secondary metabolites. They are only partly extracted during maceration and/or fermentation. Remaining seeds and skins present winery by-product which gives us an available and cheap material to work with. Bioactive compounds such as phenols are compounds that plants produce for their own protection, we can add them to increase the value of different products (e.g. food or cosmetic preparations). Recent investigations have been carried out on the content of specific secondary metabolites of certain indigenous and local Slovenian *V. vinifera* grape varieties, such as the 'Zelen'. These grape varieties are a rich source of caftaric acid, flavonoids, including quercetin derivatives, proanthocyanidins and various catechins, which are known to have a positive impact on human organism (Trošt et al. 2011).
Olive leaf extract (OLE) is a dark brown, bitter-tasting liquid derived from the leaves, an agricultural waste, of the olive tree (*Olea europaea* L., Oleaceae). OLE is marketed as a natural medicine with wide-ranging health benefits. In addition to its antioxidant properties, phenolic compounds within olive leaf extract have shown antimicrobial activities against several microorganisms. Although the individual phenolic compounds in olive leaf extract may show strong *in vitro* activities, the antioxidant and antimicrobial activities of combined phenolics showed similar or better effects than the individual phenolics. It had also been

previously supported that extracts may be more beneficial than isolated constituents, since a bioactive component can change its properties in the presence of other compounds present in the extract (Aytul, 2010).
The effectiveness of biophenols depends on preserving the stability, bioactivity and bioavailability of the active ingredients. This is a big challenge, as only a small proportion of the molecules remain available following oral administration, due to insufficient gastric residence time, low permeability and/or solubility within the gut, as well as their instability under conditions encountered in food processing and storage (temperature, oxygen, light), or in the gastrointestinal tract (pH, enzymes, presence of other nutrients), all of which limit the activity and potential health benefits of the nutraceutical components, including polyphenols. The unpleasant taste of most phenolic compounds also limits their application. The utilization of encapsulated polyphenols, instead of free compounds, can effectively alleviate these deficiencies. Microencapsulation is defined as a technology of packaging materials in miniature, sealed capsules that can release their contents at controlled rates under specific conditions (Fang and Bhandari, 2010).
Cyclodextrins (CDs) are non-branched cycloamyloses composed of α-(1→4)D-glucopiranoside units. Typical cyclodextrins, α-, β- and γ-cyclodextrin, contain six, seven or eight glucose monomer units. They have the ability to form inclusion complexes with a wide variety of organic compounds, which enter partly or entirely into the relatively hydrophobic cavity of CDs, therefore the CDs act as molecular encapsulants. Encapsulation with CDs leads to enhanced dissolution rate, membrane permeability and bioavailability of nutraceuticals of low solubility. They protect against oxidation, light-induced decompositions and heat-induced changes. CDs improve shelf life of food products and mask or reduce undesired taste (Szente and Szejtli, 2004). Possibly the most important property of encapsulation is that CDs increase the aqueous solubility of various sparingly soluble compounds (Kalogeropoulos et al., 2010; Karathanos et al., 2007). ß-cyclodextrin has been approved as GRAS to be used as a food additive.
Liposomes are constructed of polar lipids that form self-organized colloidal particles when placed in the aqueous medium. The hydrophilic interaction of the lipid head groups with water results in the formation of multilamellar and unilamellar systems (vesicles). They consists of simple lipid bilayers that resemble biological membranes, in the form of a spherical shell. Because of their entrapping ability, biocompatibility and non-toxicity, liposomes are considered as vesicles, being utilized in the entrapment, delivery, and release of water soluble, lipid-soluble, and amphiphilic materials (Vemuri and Rhodes, 1995; Keller, 2001).

MATERIALS AND METHODS

Preparation and characterization of phenolic extracts

White grape variety ‘Zelen’ (*Vitis vinifera* L.) from the Vipava Valley wine-producing region (Slovenia) was selected on the basis of previous antimicrobial testing (Smole Možina et al., 2011). The grapes were harvested at technological maturity and processed by typical vinification procedure, with only 2h of maceration. Grape seeds and skins as winery wastes after pressing were collected from a large scale press (approx. 2 kg), stored at -20 °C overnight and freeze dried. Freeze dried samples were kept frozen at -20 °C until the extraction. Prior extraction, the samples were powdered in a mortar with the liquid nitrogen and mixed with the extraction solvent (0.5g/10mL), which was in this case water:ethanol (1:1). The extraction was performed overnight including 40 min of ultrasonication in Sonorex bath (Bandelin, Germany) and prepared for further analysis as described previously (Trošt et al., 2011). The total phenolic concentration (TP) was determined by the Folin-Ciolcalteau method, calibrated against gallic acid standard and expressed in mg GAE/g of dry extract, for the sample ‘Zelen’ it was 80 mg GAE/g of extract (Trošt et al., 2011).
The olive leaf samples were taken from the cultivar ‘Leccino, harvested in 2010 from the same tree, freeze dried and pulverized. The extraction was done with ethanol:water (7:3) extraction solvent, followed by dewaxing step with the hexane and dried in rotary evaporator

at 40 °C. The extraction yield was determined gravimetrically and total phenolic content was calculated on the basis of HPLC UV-DAD response for biophenolic determination of hydroxytyrosol, tyrosol, luteolin-7-O-glucoside, oleuropein, and apigenin-7-O-glucoside. Some other non-assigned biophenols were quantified using detector response for oleuropein, as reported previously (Butinar et al., 2011). The amount of total phenolic compounds in 'Leccino' extract was 76 mg GAE/g of dry extract (Butinar et al., in press).

Encapsulation of the plant phenolic extracts

The inclusion complexes of plant extract in β-cyclodextrin (β-CD) were prepared by dispersing ethanol solution of extract in aqueous solution of β-CD in 1:2 molar ratio of total phenols to β-CD and mixed in a laboratory stirrer for 24 h at room temperature. In order to remove any insoluble monoterpenes, the suspensions were filtered through a 0.45-μm membrane filter. The filtrates were frozen at –40°C for 24 h and lyophilized. Dry β-CD-extract complex was dissolved in distilled water and filtered through 0.2-μm membrane filter in order to remove microorganisms (Mourtzinos et al., 2008).

The multilamelar liposomes (MLV) were prepared using L-α-phosphatidylcholine (Egg, Chicken; Avanti Polar Lipids, Inc. U.S.A.) and cholesterol (Sigma Aldrich, St. Louis, USA) in 5 to 1 molar ratio. Appropriate weights of lipids, dissolved in chloroform and OLE which was dissolved in etanol were transferred into 50 mL glass round-bottomed flask where solvent was evaporated under reduced pressure (17 mbar). The film was then hydrated with 2 ml of a phosphate buffersaline solution (PBS) (pH 7.4) and vigorously vortexed for 15 min. This suspension was allowed to hydrate for 2 h in the dark at room temperature in order to anneal any structural defects. The compound-incorporated MLV were separated from the unincorporated compounds by ultracentrifugation. 21% of phenolic compund was incorporated. Vesicular dispersions were spun in an ultracentrifuge at 4°C and 30,000 rpm for 60 min (Liolios et al., 2009). Since the encapsulation in β-cyclodextrin (β-CD) gave better results than MLV for olive leaves extracts, we used for grape skin extracts only β-CD encapsulation.

Antimicrobial susceptibility testing

In this study spoilage yeasts were used as target organisms. We tested 14 strains belonging to genera *Candida, Pichia*, *Ogataea* and *Dekkera bruxellensis,* which were isolated either from spoiled olive oil or grape must, respectively. They were identified on the basis of their physiological characteristics and by sequencing of LSU rRNA gene (Cadez *et al.,* in press). They were deposited in Collection of Industrial Microorganisms (ZIM, Slovenia). Yeast cultures were incubated for 24-48 h in YPD broth (Sigma), and properly diluted for further testing.

Broth microdilution method was used to determine minimal inhibitory concentrations (MICs) of all the extracts tested against selected target yeasts. The tests were performed in sterile 96-well microtitre plates already containing 50 μL of two-fold serially diluted extracts. The concentrations of total phenols ranged from 10 - 0.06 mg GAE/mL YPD. The final volume in each well was 100 μL, the contents were mixed on a microplate shaker (Eppendorf, Hamburg, Germany) at 900 rpm for 1 min prior to incubation for 24 h at 37 °C. The MIC was defined as the lowest concentration where no viability was observed after 24 h. The presence of the purple colour, as indicator of the respiratory activity, was determined visually after adding 10 μL $well^{-1}$ of yeast growth indicator p-iodo-nitro tetrazolium violet (INT, Sigma) and incubated at 37 °C for 30 min in dark. MICs were tested in triplicate and given in the concentration where no metabolic activity was most often observed. The kinetics of microbial inhibition was evaluated using the broth macrodilution method where the extracts were added to the growth medium (5 mL) in concentrations equal to MICs determined in broth microdilution test as described earlier (Klančnik et al. 2010). Microbial growth inhibition was followed by sampling during 5 days of incubation in two replicates. The samples were taken, diluted and cultured on YPD agar in order to determine the cell concentration. The mean log CFU mL^{-1} were presented.

RESULTS AND DISCUSSION

Antimicrobial activity of the phenolic extracts obtained from the grape seeds and skins after vinification process as well as from the olive leave extracts was first screened by broth microdilution test and expressed in minimal inhibitory concentrations (in gallic acid equivalents (GAE) of total phenolic compounds in these extracts. They differed significantly in qualitative and quantitative composition of phenolics and reached for olive leaves extracts the MIC values from 0.19-3.0 mg GAE/ml for different spoilage yeasts isolated from olive oil and wine. For the extracts from grape skins and seeds (GSSE) the MIC values were from 0.008-3.0 mg GAE/ml. In Table 1 the activity is expressed for comparative analysis of the non-capsulated extracts as well as for the capsulated extracts in β-cyclodextrin (β-CD) and also MLV (only in case of OLE), where the encapsulation resulted in improved antimicrobial efficiency (Table 1).

Table 1. Antimicrobial activity of the phenolic extracts from olive leaves (OLE) and grape skins and seeds (GSSE), expressed as MICs (in mg of GAE of total phenols/ml of the medium. MICs of the encapsulated extracts (in CD and liposomes) are presented as well.

	Candida tenuis **ZIM 2324**	***Candida molendinolei*** **ZIM 2329**	***Dekkera bruxellensis*** **ZIM 701**	***Ogatea dorogensis*** **ZIM 2322**
OLE	0.38	0.75	0.19	0.75
OLE – β-CD	0.064	0.25	0.13	n.d.
OLE - MLV	0.085	0.34	0.17	0.17
	Candida molendinolei ZIM 2280	*Candida adriatica* ZIM 2334		
GSSE	0.12	0.12	1.5	0.004
GSSE – β-CD	0.07	0.14	2.2	0.009

Phenolic extracts from olive leaves and grape skins and seeds in general expressed high efficiency in growth inhibition of most of the spoilage yeasts tested. MICs values were comparable to the results collected in a similar way on bacterial pathogens as target organisms (Katalinić et al., 2010; Trošt et al., 2011).

Encapsulated GSSE and OLE in ß-cyclodextrin and in liposomes (only in case of OLE), have shown similar or better efficiency than non-capsulated in broth microdilution screening test (Table 1). OLE and GSSE which are normally hydrophobic after encapsulation become soluble in water. Lipid bilayer of liposomes and hydrophobic cavity of β-CD in which hydrophobic phenol compounds are placed make this phenomenon possible. Due to encapsulation, contact of active compounds and cells was better and therefore bioavailability increased.

According to the most efficient antimicrobial activity expressed in low MICs in broth microdilution test we selected combinations of yeast and extract preparations for further testing of kinetics of growth inhibition in broth macrodilution test with prolonged incubation time. In case of olive leaves extract we compared the activity of control culture with antimicrobial additives as well as non-capsulated extract (OLE), and the extract prepared in liposomes (OLE-LP) and in ß-cyclodextrin (OLE-CD) (Figure 1), but in case of grape seed extracts we selected *Candida adriatica* ZIM 2334 (Figure 2a) and *Candida molendinolei* ZIM 2280 (Figure 2b) to test non-capsulated extract (GSSE) and encapsulated in ß-cyclodextrin (GSSE-CD).

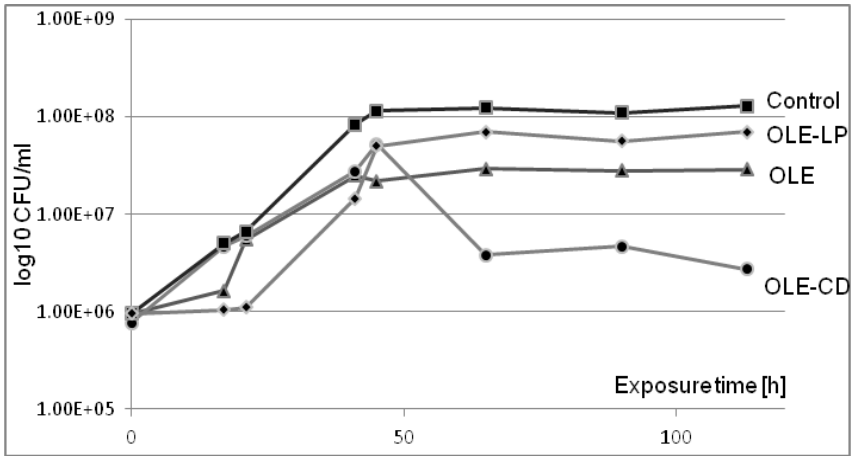
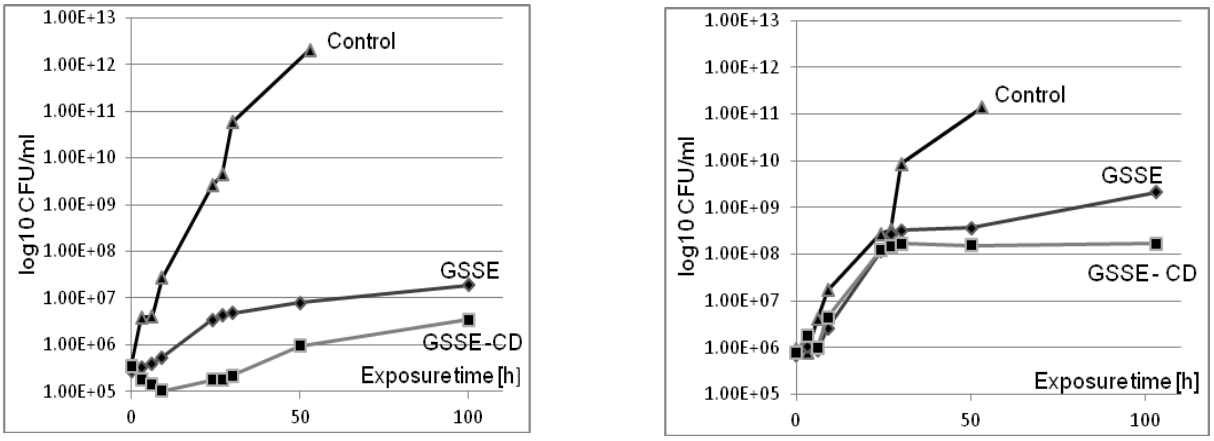


Figure 1. Growth inhibition curves of the yeast *Dekkera bruxelensis* after exposure to olive leaves phenolic extracts at concentrations found as MICs in broth microdilution test (0.13-0.19 mg GAE/mL).



a) **b)**

Figure 2. Growth inhibition curves of the yeast *Candida adriatica* (a) *Candida molendinolei* (b) after addition of grape seeds and skins extracts (GSSE) in YPD at concentrations found as MICs in broth microdilution test (0.12-0.14 and 0.07-0.12 mg GAE/mL, for non-capsulated (GSSE) and encapsulated (GSSE-CD) extracts, respectively).

During prolonged cultivation encapsulated extracts showed better inhibition properties (Fig. 1,2). This may be due to the protective ability of liposomes and β-CD, which protected sensitive phenolic compounds from degradation caused by oxygen, light, temperature, etc. We showed that the encapsulation into β-CD is more efficient than into the liposomes. Better stability of capsules of β-CD than liposomes may be the reason why the extracts encapsulated in β-CD have shown better results in both microdilution test and kinetics of antifungal activity.

CONCLUSIONS

Grape skins and seeds and olive leaves as plant waste materials have been proven as a rich source of phenolic compounds with antimicrobial activity against spoilage yeasts. In addition, a positive impact of encapsulation was confirmed with preserved or enhanced antimicrobial activity of tested phenol extracts against spoilage yeasts. Considering demands of consumers to use natural preservatives in food products, these results provide a solid basis for further research in order to use plant extracts as food preservative.

ACKNOWLEDGEMENTS

The authors are grateful to Slovenian Research Agency (ARRS) for financial support of the project V4-1079 as well as for CEEPUS grant to D.T. that enabled this study.

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CHARACTERISTICS OF KOMBUCHA FERMENTED MILK PRODUCTS WITH PEPPERMINT

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ABSTRACT: The aim of this study was to investigate characteristics of fermented milk products, obtained from milk with 2.8% milk fat, by using two starter cultures at 37, 40 and 43°C. One starter culture was kombucha fermentative liquid, obtained from peppermint extract sweetened with sucrose (7%), and the other was a mixture of kombucha fermentative liquid on peppermint and probiotic yoghurt culture. The quality of the products was followed by monitoring of chemical composition, physico-chemical characteristics, syneresis, water holding capacity and sensory characteristics of the products after fermentation and after 10 days of storage. Fermentation performed using a mixture of fermentative liquid of kombucha on peppermint and probiotic yoghurt culture was significantly shorter in comparison to the other starter culture, at all temperatures. It was the only major difference because the quality of the obtained products was very similar regardless of the fermentation temperature, applied starter culture and storage.
Key words: *kombucha, fermented milk products, peppermint*

INTRODUCTION

Kombucha is a symbiotic association between yeasts and acetic acid bacteria. It is capable to convert the simple substrate, usually black or green tea sweetened by sucrose, into a pleasant, refreshing and slightly carbonated beverage. On this substrate, fermentation lasts about 7 days, on temperature 20-30^{o}C. It was found the possibility of obtaining of fermented milk beverages by means of kombucha (Malbaša et al., 2009).
Using an appropriate technological process, it is possible to produce fermented milk products from milk with 1.5% milk fat, with the addition of 10% kombucha inoculum cultivated on herbal extracts (winter savory, peppermint, wild thyme and stinging nettle) at 42^{o}C. The characteristic pH value for this type of products is 4.5. The fermentations with inoculums containing wild thyme and stinging nettle lasted for approximately the same time (10.5 and 11 h, respectively), whilst for fermentations with inoculums containing winter savory and peppermint this time was 13 and 17 h, respectively. Chemical composition and physico-chemical characteristics of the obtained products corresponded to the current Regulation on quality of yoghurt and kefir. The highest value of water holding capacity, the lowest value of syneresis and the best textural characteristics showed sample produced with inoculum containing peppermint. This indicated on better quality of this product compared to others (Vitas et al., 2011).
However, the best characteristic of the products containing peppermint in previous investigations (Vitas et al., 2011), inspired the further investigations with kombucha inoculum with peppermint. The intention was the shortening of the fermentation time. Some of the process conditions like fermentation temperature, milk fat in substrate, kombucha inoculum containing peppermint were changed.
The aim of this study was to investigate characteristics of fermented milk products, obtained from milk with 2.8% milk fat, by using two starter cultures at 37, 40 and 43°C. One starter culture was kombucha fermentative liquid, obtained from peppermint extract sweetened with sucrose (7%), and the other was a mixture of kombucha fermentative liquid on peppermint and probiotic yoghurt culture. The products quality was monitored using typical chemical,

physico-chemical and sensory analyses after fermentation and after 10 days of storage, as well as comparison with commercial probiotic yoghurt (Y) and kefir (K).

MATERIAL AND METHODS

Inoculums

Two types of inoculums were used for production of fermented milk products.
The first inoculum was fermentation liquid obtained by cultivation of kombucha on cooled tea, which was prepared as follows: in 1 L of boiling tap water was added 70 g sucrose and 2.25 g of peppermint tea. Prepared tea has cooled to room temperature, strained and then added 100 mL of inoculum from a previous fermentation. A glass jar was covered with fabric bandwidth. Kombucha incubation was performed at room temperature for 7 days.
The other inoculum was a combination of starters: 10% kombucha inoculum on peppermint and 0.01% probiotic ABT-7 culture (Probiotic culture-Probio-Tec®Contains BB-12®, CHR Hansen, Denmark).

Production of fermented milk products

A pasteurized, homogenized milk with 2.8% milk fat, from the manufacturer ››AD IMLEK‹‹ Beograd, branch „Novosadska mlekara“, Novi Sad, was used for the production of fermented milk products in the laboratory.
Amount of 10% (v/v) of kombucha inoculum on peppermint (P) and a combination of starters (PP) was added to milk with 2.8% milk fat. Fermentation was performed at 37, 40 and 43°C until pH value of 4.5 was reached. Gel was then cooled to the temperature of 8°C and homogenized by mixer. The obtained samples were labeled with P37, P40, P43, PP37, PP40 and PP43. The labels indicate used inoculum as well as fermentation temperature.

Methods of analysis

The milk, which was used for the production of fermented milk products, the obtained fermented milk products, commercial probiotic yoghurt (manufacturer Somboled, Sombor), and kefir (manufacturer ››AD IMLEK‹‹ Beograd, branch „Novosadska mlekara“, Novi Sad) were analyzed by measuring the pH, acidity, dry matter, ash, milk fat, total protein and lactose, according to standard methods (Carić et al., 2000).
Syneresis of whey (Atamer et al., 1996) and water holding capacity (WHC) (Guzman-Gonzalez et al., 1999) of laboratory-obtained fermented milk products, commercial probiotic yoghurt and kefir were determined, and given their sensory evaluation.
All fermented milk products were analyzed after production and after 10 days of storage.

RESULTS AND DISCUSSION

Analysis of milk

The results of the quality of milk used in the production of kombucha fermented milk products are given in Table 1.
Based on the results given in Table 1 it was found that the characteristics of milk correspond to the current Regulation on quality of milk products and starter cultures.

Table 1. Chemical composition and physico-chemical characteristics of milk

characteristic	milk
pH	6.61
dry matter (%)	11.50
ash (%)	0.72
acidity (˚SH)	6.40
milk fat (%)	2.80
total proteins (%)	3.17
lactose (%)	4.60

Relative standard deviation (RSD) for all measurements was up to 5%.

Changes of pH during fermentation of milk

Course of fermentation of milk with 2.8% milk fat in the production process of kombucha fermented milk products is shown in Figure 1.

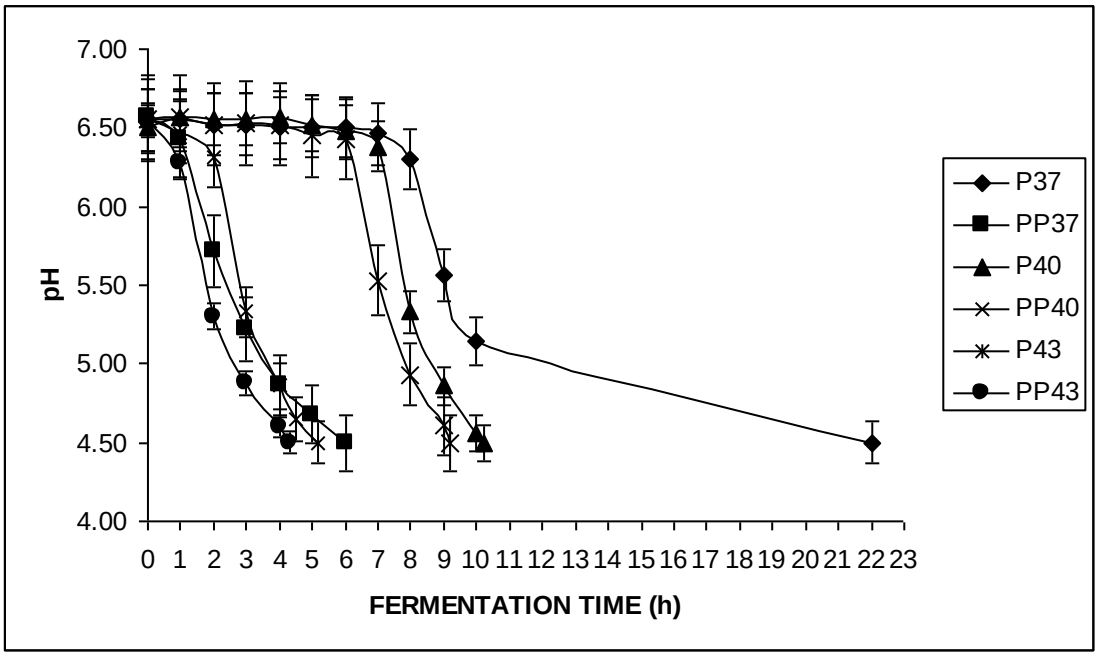

Figure 1. Fermentation process of kombucha fermented milk products

Fermentation was stopped after reaching the pH value of 4.5. With temperature increase, time of fermentation process was shorter, with all applied inoculums. Combination of starters (PP) shortened the fermentation process significantly, compared to P inoculum, at all applied temperatures.

Significant changes in pH values for sample P37 was recorded after 10h of fermentation, whilst for samples P40 and P43 these changes occurred after 8h of fermentation. For samples PP37 and PP40 significant changes in pH values occurred after 3h of fermentation, and for sample PP43 these changes occurred after 2h of fermentation.

The patterns of decrease in pH value during fermentation of P37, P40 and P43 were typical for kombucha fermentation on milk, i.e. the curves were sigmoidal. The fermentation curves for PP37, PP40 and PP43 were almost linear which is usual for yoghurt (Malbaša et al., 2009). It indicates that the probiotic bacteria took major role during the fermentation of combination of starters on milk.

Fermentation was the longest for sample P37 (22h), and shortest for sample PP43 (4.30h).

Analysis of kombucha fermented milk products

The chemical composition and physico-chemical characteristics of kombucha fermented milk products after production is shown in Table 2.

Table 2. Chemical composition and physico-chemical characteristics of kombucha fermented milk products after production, yoghurt and kefir

characteristic	P37	PP37	P40	PP40	P43	PP43	Y	K
pH	4.10	4.05	4.10	4.33	4.17	4.11	3.96	4.22
acidity (^{o}SH)	40.20	42.20	38.00	40.00	36.00	35.80	39.20	37.80
dry matter (%)	10.95	10.62	11.18	11.04	11.29	10.97	11.12	11.24
ash (%)	0.65	0.64	0.65	0.63	0.66	0.63	0.69	0.71
lactose (%)	3.91	3.93	3.98	3.88	3.62	3.95	4.29	3.86
total proteins (%)	3.18	2.97	3.00	2.99	3.03	2.98	3.41	3.15
milk fat (%)	2.64	2.64	2.64	2.64	2.64	2.64	2.75	2.53
syneresis (mL)	24.50	20.00	29.00	31.00	32.00	30.00	26.00	25.00
WHC (%)	66.05	49.50	32.84	42.79	41.00	39.50	52.24	71.50

Relative standard deviation (RSD) for all measurements was in a range from 3 to 12%.

The laboratory-produced samples with the addition of P showed higher dry matter content compared to the ones produced with the addition of PP, at all applied temperatures. The highest value of dry matter and ash content of samples obtained in the laboratory showed P43, 11.29% and 0.66%, respectively. Sample P43 showed higher dry matter content compared to Y and K. Ash content of all laboratory-produced samples was lower compared to Y and K.

In all laboratory-produced samples was determined the same percentage of milk fat, which was 2.64%. Y and K showed milk fat content which was in accordance to the value stated on the declaration of the product packaging. The content of total proteins for all laboratory-produced samples was in narrow interval and ranged from 2.97 (PP37) to 3.18% (P37). Protein content for Y and K was in accordance to values given on product declaration. The lowest value of lactose content showed sample P43. This value for other laboratory produced samples ranged from 3.88 (PP40) to 3.98% (P40).

Acidity of all samples was characteristic for this type of products and ranged from 35.80 (PP43) to 42.20^{o}SH (PP37).

pH value of all samples was in accordance with current Regulation for yoghurt and kefir.

Compared to K all laboratory-produced samples showed lower WHC. The highest water holding capacity of laboratory-produced samples showed sample P37 (66.05%) and this indicates the better quality of this product comparing to the other laboratory-produced samples, and including Y. This value is also higher than value obtained for set yoghurt made from goat milk (Vučić et al., 2010).

The values of syneresis of whey of laboratory-produced samples were in narrow interval and ranged from 20.00 mL for PP37 to 32.00 mL for P43. These values are in accordance with experimentally determined limits, for fermented milk beverages obtained by applying kombucha cultivated on black tea (Malbaša et al., 2009), kombucha cultivated on stinging nettle and combination of kombucha on stinging nettle and probiotic starter culture (Lončar et al., 2011). Samples P37 and PP37 showed lower value of syneresis comparing to Y and K.

Table 3. shows the chemical composition and physico-chemical characteristics of kombucha fermented milk products after 10 days of storage.

P40 and P43 showed higher dry matter content compared to P37, PP37, PP40 and PP43. The highest value of dry matter content of samples obtained in the laboratory showed P40 (11.35%). Laboratory-produced samples, except P37, showed higher dry matter content compared to Y. Sample K showed the highest dry matter content. Ash content of all laboratory-produced samples was lower compared to Y and K.

Table 3. Chemical composition and physico-chemical characteristics of kombucha fermented milk products, yoghurt and kefir after 10 days of storage

characteristic	P37	PP37	P40	PP40	P43	PP43	Y	K
pH	4.09	4.00	4.10	4.11	4.15	4.15	4.08	4.33
acidity (^{o}SH)	40.20	47.60	41.40	46.00	40.60	37.20	39.80	41.80
dry matter (%)	10.96	11.10	11.35	11.18	11.34	11.19	11.07	11.38
ash (%)	0.62	0.64	0.65	0.63	0.65	0.63	0.67	0.71
lactose (%)	2.50	3.27	2.98	3.34	3.34	3.34	3.64	3.32
total proteins (%)	2.95	2.89	3.11	3.04	3.06	3.05	3.39	3.17
milk fat (%)	2.64	2.64	2.64	2.64	2.64	2.64	2.86	2.53
syneresis (mL)	30.00	28.00	29.00	30.00	32.00	30.00	26.00	25.00
WHC (%)	55.22	48.00	38.50	44.28	37.00	38.69	57.71	55.50

Relative standard deviation (RSD) for all measurements was in a range from 2.5 to 10.5%.

In laboratory-produced samples the percentage of milk fat did not changed during the storage period. Y and K showed milk fat content which was in accordance to the value stated on the product declaration. The content of total proteins for all laboratory-produced samples was in narrow interval and ranged from 2.89 (PP37) to 3.11% (P40). Values of protein content of Y and K were in accordance to product declaration. The lowest value of lactose content (2.50%) showed sample P37. This value for other laboratory-produced samples ranged from 2.98 (P40) to 3.34% (PP40, P43 and PP43). The lactose content of all samples decreased during storage period of 10 days.

Acidity of samples increased during storage. For sample P37 this value did not changed.

pH value of all samples was in accordance with current Regulation for yoghurt and kefir.

After storage, all laboratory-produced samples showed lower WHC compared to Y and K. The highest water holding capacity of laboratory-produced samples showed P37 (55.22%). WHC of P40, PP40 and Y increased during storage.

The values of syneresis of whey of laboratory-produced samples were in narrow interval and ranged from 28.00 mL for PP37 to 32.00 mL for P43. The values of syneresis for samples P37 and PP37 increased during storage, and decreased for sample PP40. This value did not change for samples P40, P43, PP43, Y and K.

Sensory analysis of kombucha fermented milk products

Sensory evaluation of produced fermented milk products and commercial yoghurt and kefir was obtained by qualified assessors.

After production, assessment noted that all laboratory-produced samples did not have separated whey on the surface; their color and consistency were typical for that type of product. Samples P37, PP37 and P40 had bitter taste, whilst samples PP40, P43 and PP43 showed mild and pleasant taste, which is typical for fermented milk beverages obtained by applying kombucha cultivated on peppermint extract. In addition, samples produced with PP addition were more sourly comparing to samples produced with P addition. Y and K showed sensory characteristics that were in accordance to the current Regulation.

After 10 days of storage, assessment established that all laboratory-produced samples did not have separated whey on the surface; their color and consistency were typical for that type of product. All of these samples showed bitter taste which was slightly less pronounced in the case of P40, PP40, P43 and PP43. Characteristic odor on peppermint extract was not determined in the case of P37, PP37 and P40, and in the case of PP40, P43 and PP43 it was slightly pronounced. Bitter taste after storage (Table 3.) could be the consequence of higher acidity. Y showed sensory characteristics that were in accordance to the current Regulation, whilst K had bitter taste.

CONCLUSIONS

Using an appropriate technological process, fermented milk products were produced from milk with 2.8% milk fat, with the addition of 10% kombucha inoculum cultivated on peppermint extract and with the addition of combination of starters.
Combination of kombucha and probiotic starters significantly shortened the fermentation.
All products were in accordance to actual Regulation.
After 10 day storage the taste was less or more bitter as a consequence of higher acidity.

ACKNOWLEDGEMENT

Authors want to thank to Ministry of Education and Science of Republic of Serbia for the finansing of investigations presented in this article, Grant III-46009.

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COMPARATIVE STUDY OF BREWING YEAST STRAINS FOR BEER PRODUCTION WITH IMMOBILIZED CELLS

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ABSTRACT: In addition to raw materials, wort, fermentation conditions, methods of stabilization, and technological equipment, brewing yeasts have also a significant impact on the flavor profile of beer. Brewing yeasts with good reproductive capacity, fermentation activity, metabolism and stable properties are crucial for producing competitive beers with high quality. Therefore, an important step in the development of a new technology or the improvement of an existing one is the selection of yeast strains.
In the present study three dry brewing yeasts strains – two lager strains and an ale one were investigated. Fermentation processes were carried out with free and immobilized cells at different initial wort extracts. Yeast immobilization was carried out in the capsules of chitosan-alginate with an internal liquid core. Some basic parameters of produced beers were investigated and an initial organoleptic evaluation of the beverages was made. All analyses were performed according to the standard methods of brewing technology and microbiology. As a result of the study, yeast strains suitable for beer production with immobilized cells were selected.
Key words: *brewing yeast, immobilization, beer quality*

INTRODUCTION

The conventional brewing process consists of four stages: malting, wort production, fermentation, and beer stabilization together with filtration. The purpose of malting is to produce enzymes in the barley kernel, which will cause defined changes in its substances.The purpose of wort production is to transform and dissolute the insoluble components of the malt into soluble and particularly fermentable sugars. During fermentation wort transforms into beer under the action of active yeast cells inoculated into the cooled and aerated wort. The filtration and stabilization of the beer is carried out in order to achieve microbial, colloidal, and flavour stability so that beer quality remains unchanged for a long time (Branyik et al., 2005).
During fermentation and maturation yeasts not only produce alcohol and CO_2 but also significantly affect the taste and character of beer with their metabolism. Brewing yeasts are classified as: ale strains (Saccharomyces cerevisiae) and lager strains (Saccharomyces pastorianus) according to their behaviour during fermentation. Ale strains rise to the surface during fermentation, while lager strains settle to the bottom at the end of fermentation (Kunze, 2004). Lager strains are capable of utilizing disaccharide melibiose and exhibit a greater affinity for galactose and maltotriose than ale strains. Lager strains are better 'equipped' to grow at lower temperatures, and perform fermentation at lower temperatures than ale strains (Boulton and Quain, 2001). The products of yeast metabolism, which affect beer quality, are: vicinal diketones, aldehydes, esters and higher alcohols. Their production is different for ale and lager strains. Ale strains produce more fusel alcohols and esters than lager ones. The proportion of these fermentation by-products is up to 50 % more than in the case of bottom fermentation (Kunze, 2004). The ales flavor is usually described as "robusty, hearty and fruity". Lagers are characteristically "smooth, elegant, crisp, fruity, and clean".
Fermentation is the most time-consuming step in overall beer production. In such a competitive market, the potential time savings offered by immobilized cell technology (ICT) has to be taken into account. The immobilization of microbial cells includes any technique

that limits their free migration (Masschelein et al., 1994). Cell immobilization methods can be classified into four main categories: (i) attachment to the surface of solid carriers; (ii) entrapment within a porous matrix; (iii) self-aggregation naturally or artificially induced; (iv) containment behind barrier (Nedovic et al., 2005). Microencapsulation is an interesting variation of standard containment behind barrier method. Cells are usually entrapped in gel, coated with a polymer such as chitosan, and then, after the gel is dissolved, the cells are contained behind polymer barrier (Masschelein et al., 1994). The main challenge for fermentation with immobilized cells is to reproduce the traditional beer flavor. The differences between beers produced conventionally and beers produced using ICT were discussed in many papers (Willaert and Nedovic, 2006; Branyik et al., 2008).
In this study we compared the effect of immobilization on the fermentation activities of three brewing yeast strains and on the quality of the final product. The choice of yeast strain, suitable for beer fermentation was based on the comparison of some basic parameters such as: extract, alcohol content, and degree of attenuation. On the other hand, the initial organoleptic evaluations of the produced beers, made by experienced tasters were also crucial for the yeast choice.

MATERIAL AND METHODS

Yeast strain

Three dry brewing yeast strains were used – two lager strains: Saflager S-23 and Saflager W/34-70 and one ale strain: Safbrew S-33. They were re-hydrated in sterile water (10 times their own weights) at temperature 23±3°C for lager strains and 27±3°C for the ale one before the immobilization.

Cell immobilization

The cells were immobilized in 3 % calcium alginate gel. Sodium alginate (Algogel 6021) was purchased from “Degussa” (France). After having autoclaved the alginate solution for 20 min at 120°C, the latter was mixed with the cell suspension to obtain a cell concentration of 10^7 cells/mL of gel. This suspension was forced through a syringe needle by means of peristaltic pump and dropped into 2% (w/v) $CaCl_2$ solution. The resulting beads were approximately 2 mm in diameter. The beads were left for 30 min in calcium chloride solution and then were placed into 0.38% (w/v) chitosan solution in 1% acetic acid (v/v). Alginate beads stayed in chitosan solution for 60 min. Afterwards, chitosan-alginate beads were washed with sterile water in order to remove the excess of chitosan. Beads stayed in 0.05 M sodium citrate solution for 30 min to construct microcapsules with liquid core. Alginate gel is sensitive towards chelating agents like citrate, so its property is of key importance for dissolving alginate and producing capsules with liquid core. Chitosan-alginate microcapsules were stored in physiological solution (saline) before use.

Wort

Wort supplied by a local brewery with extract of 12% and 17% was used. It was autoclaved at 120°C for 20 min.

Fermentation

7 g microcapsules were added to 200 ml sterile wort. Free cell fermentation process was used as a control probe for fermentation rate. The free cells suspension, containing 10^7 cells/mL was added to 200 ml sterile wort. The fermentation was carried out in fermentation bottles, equipped with airlock system. The fermentation processes were monitored via the amount of released CO_2 which was measured from the weight loss. The fermentation was carried out in temperature controlled room at 15 °C.

Analytical methods

Extract, alcohol content, and degree of attenuation of produced beers were analyzed according to the EBC methods of analysis (Analytica - EBC, 2004). The biomass

concentration was measured at OD 600. Microcapsules were dissolved in 1 M magnesium citrate solution. The solution was centrifuged at 3000 rpm for 10 min. The biomass was washed with saline twice and OD 600 was measured by „Shimatzu UV-VIS 1800". The results were converted to the corresponding g dry weight/l using a standard curve according to Zhou et. al., 1998.

RESULTS AND DISCUSSIONS

The results for fermentation activities of free and immobilized cells of yeast strain Saflager S-23 are presented in Figure 1. The fermentation proceeded normally, regardless of the original gravity of wort. The fermentation activities of free and immobilized yeast cells reached their maximum between 2nd and 3rd day. Afterwards, they began to fluently decrease. When immobilized cells were used, shifted maximum of fermentation activity was observed. The main reason for the latter is that microencapsulation makes CO_2 release more difficult. The increase of original gravity of wort led to an increase in the observed shift. However, the fermentation proceeded without significant modifications. The amount of non-fermented extract was higher for free cells, which led to lower real degree of attenuation of produced beer.

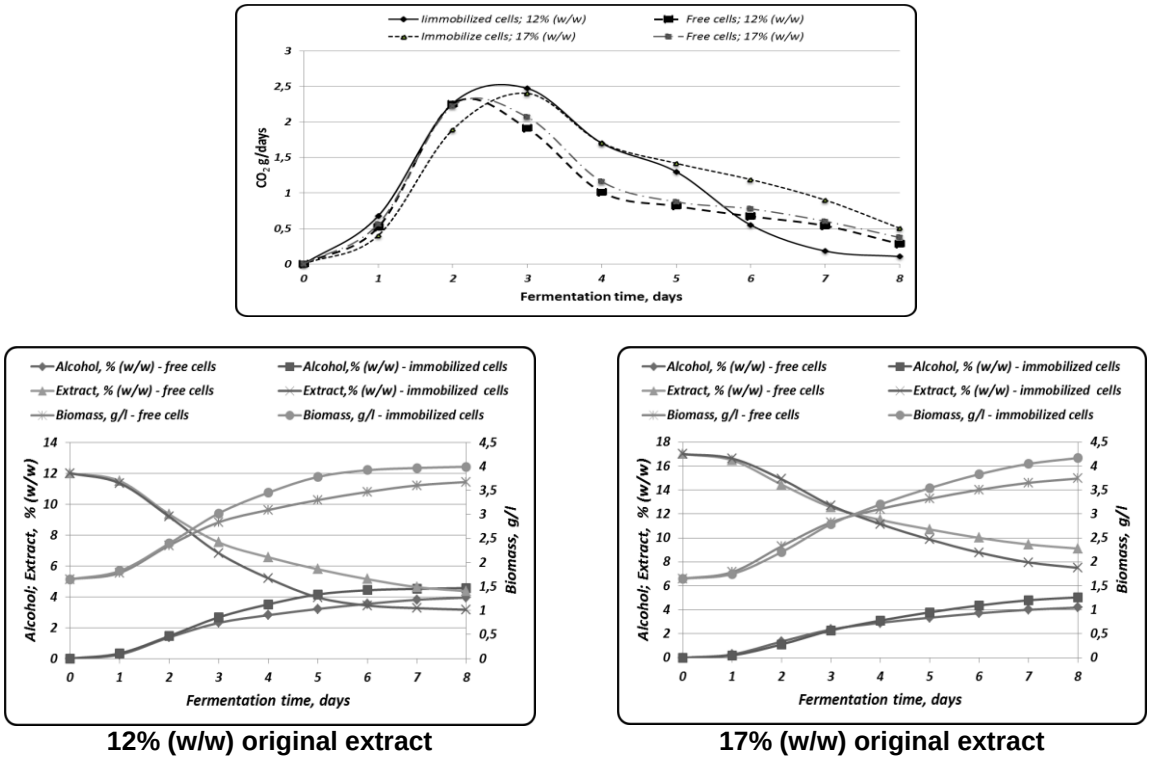
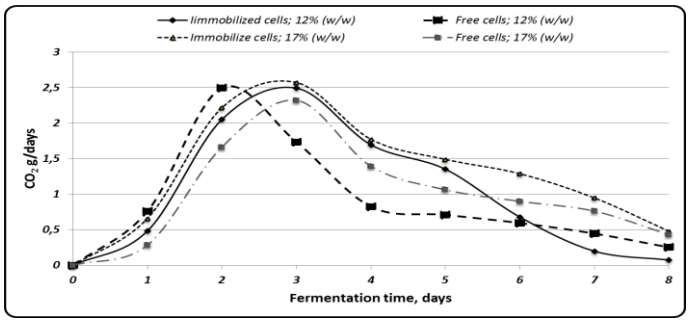
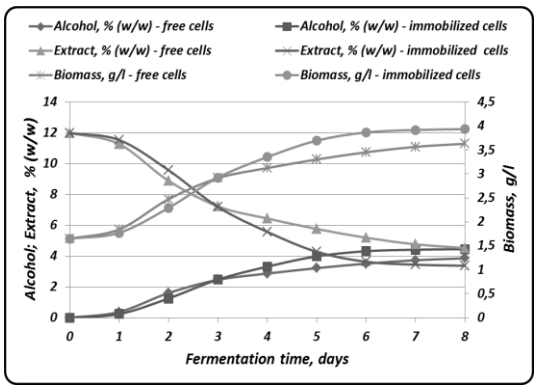
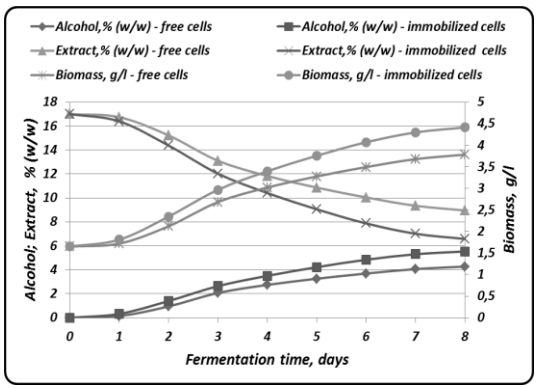


Figure 1. Fermentation activity of yeast strain Saflager S-23

Fermentation activity of yeast strain Saflager W/34-70 is shown on figure 2. The fermentation activity of both immobilized and free cells was almost the same for wort with original extract of 12%. When the original gravity of wort increased, the difference between the fermentation activities of corresponding cells also increased. Comparison of fermentation activity indicated shifted maximum in the case of immobilized yeast. Immobilized yeast showed higher real degree of attenuation compared to free cells.

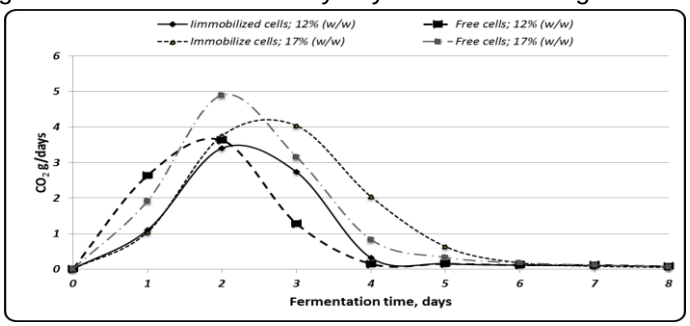


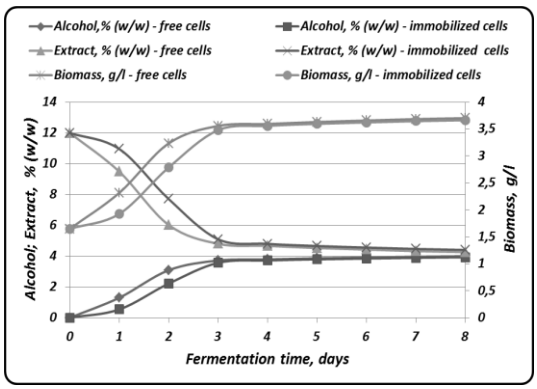


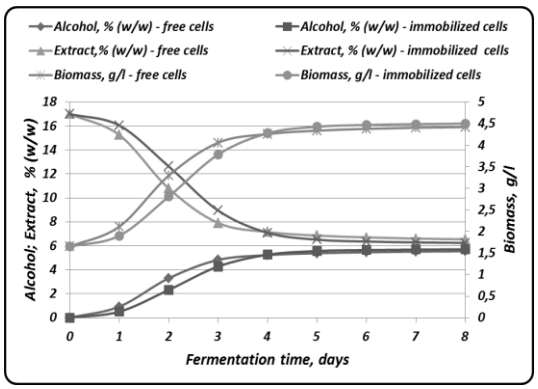


12% (w/w) original extract **17% (w/w) original extract**

Figure 2. Fermentation activity of yeast strain Saflager W34/70







12% (w/w) original extract **17% (w/w) original extract**

Figure 3. Fermentation activity of yeast strain Safbrew S-33

Fermentation activity of yeast strain Safbrew S-33 is shown on figure 3. The differences between free and immobilized yeasts were more significant for that yeast strain. The maximums of fermentation activity for all investigated variants were in different periods of time. The strain is potentially interesting for study because significant differences in fermentation processes with free and immobilized cells were observed. The main reason is that yeast immobilization affects the yeast growth and consequently the fermentation process. Furthermore, few studies on the immobilization of ale strains are represented in the literature.

Table 1 shows results for extract, alcohol, and real degree of attenuation of beers at 8th day of fermentation. Analyses of all beers showed higher degree of attenuation for beers produced by immobilized cells. The highest degree of attenuation was achieved with yeast strain Saflager S-23 when the original gravity of wort was 12 %. The increase of the original gravity of wort led to the decrease of the degree of attenuation.

Table 1. Effect of original gravity of wort and type of yeast on primary fermentation parameters of different yeast strains

Original gravity of wort	Yeast strains	Type of yeast	Apparent Extract % w/w	Real Extract % w/w	Alcohol % w/w	Real degree of attenuation %
12%	S-23	immobilized	2.23	4.24	4.36	**68.00**
		free	3.06	4.93	4.06	62.99
	W-34/70	immobilized	2.31	4.31	4.35	**67.59**
		free	3.15	5	4	62.31
	S-33	immobilized	3.03	4.79	3.82	**62.24**
		free	3.24	5.05	3.93	61.66
17%	S-23	immobilized	5.16	7.45	5.1	**58.59**
		free	6.42	8.46	4.5	52.36
	W-34/70	immobilized	3.99	6.49	5.58	**63.99**
		free	6.16	8.26	4.64	53.72
	S-33	immobilized	4.53	7	5.53	**62.01**
		free	4.63	7.08	5.5	61.62

In addition to fermentation activity, the flavor profile of beer is crucial for the choice of yeast strain for beer fermentation. Experienced tasters carried out descriptive sensorial tests in order to determine the taste and aroma profile of beers produced by ICT and conventional technology. The results are shown in Table 2. Beers produced by yeast strain Safbrew S-33 received the highest sensory evaluations. The sensory profiles of beers produced by free and immobilized yeast Safbrew S-33 revealed minor differences when wort with original gravity of 17% was used. The differences in flavor profiles of beers became more significant when the original gravity of wort decreased. The lowest sensory evaluations received all beers produced by yeast strain Saflager W 34/70.

Table 2. Descriptive sensorial tests of beers produced by immobilized and free yeast strains

Original gravity of wort	Yeast strains	Type of yeast	Description of aroma and flavor of produced beers
12%	S-23	immobilized	fruity, phenolic
		free	fruity, sweet, honey
	W-34/70	immobilized	phenolic
		free	malty, musty
	S-33	immobilized	cooked-vegetable
		free	oxidized, butterscotch, full and harmonic body
17%	S-23	immobilized	malty flavor and aroma
		free	sour-acidic odor
	W-34/70	immobilized	swampy odor, malty taste
		free	sour, unpleasant aroma
	S-33	immobilized	fruity, malty
		free	fruity – banana, apple, pear, sweet

CONCLUSION

Two lager yeast strains and an ale one were compared for beer production with immobilized cells. The beers were produced with wort of different original gravity. The ale strain showed the highest fermentation activity and initial sensory evaluation, but also the lowest degree of attenuation. The lager strains revealed minor differences in fermentation activities, but significant differences in sensorial evaluations. The beers produced by yeast strain Saflager W 34/70 were found unacceptable in flavor and aroma by experienced tasters. Acceptable and balanced beer flavor is an important prerequisite for producing competitive beers. Therefore, yeast strain Saflager W 34/70 seems not suitable for production of high quality beers.
Yeast strains Safbrew S-33 and Saflager S-23 were chosen for further studies on beer production with immobilized cells. The effect of temperature and original gravity of wort on the yeast metabolism and beer quality will be investigated in more detail and one of the yeast strains will be chosen for batch and continuous fermentation with immobilized yeast.

ACKNOWLEDGEMENTS

We would like to thank to the Bulgarian brewery “Kamenitza” Plc for generously supplying us with the wort, needed for our experiments.

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DARK CHOCOLATE AS A MATRIX FOR PROBIOTIC BACTERIA

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ABSTRACT: Food products containing beneficial microorganisms, mostly lactic acid bacteria which effectively affect human digestive and immune systems, represent one of the largest market of functional foods, with dairy products as the most accessible ones. However, for some, non-negligible percentage of the global consumer's population, usage of dairy products is limited due to diet (vegetarian), traditional reasons, or because of lactose intolerance, cholesterol content and allergenic milk proteins. These major drawbacks related to the intake of dairy products promote the idea of replacement of milk with alternative delivery formats for probiotics, which makes the development of new nondairy probiotic foods, that can appeal to a wider range of consumers, essential. In this context, our idea was related to the incorporation of probiotic microorganisms into one of the most popular foods - chocolate, which significantly contributes to the favorable impact of this product on human health. Therefore, in this study addition of probiotic strain *Lactobacillus acidophilus* NCFM® in dark chocolates with 44 % cocoa solids, was carried out in industrial conditions. In order to determine the shelf-life of these chocolates their sensory properties (appearance, texture, aroma) were evaluated, along with the determination of viability of added probiotic bacteria during storage at different temperatures (4ºC and 22ºC), immediately after production and after 90 and 180 days. Results indicated that the enrichment of dark chocolates with *Lactobacillus acidophilus* NCFM® caused no modification of the their sensory properties, and the number of incorporated live probiotic bacteria maintained at a high level of 8 log cfu/g during storage of 180 days. Based on the above it can be concluded that dark chocolates with 44 % cocoa solids have a protective effect on *Lactobacillus acidophilus* NCFM® cells and make a good matrix for the preparation of functional probiotic products.
Keywords: *dark chocolate, Lactobacillus acidophilus, probiotics, sensory properties*

INTRODUCTION

According to the definition given by FAO/WHO (2001) probiotics are „live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Most commonly this refers to certain strains of lactic acid bacteria (LAB), particularly from the genera *Lactobacillus* and *Bifidobacterium.* Their impacts are primarily associated with maintaining or improving the balance of intestinal microflora (Fuller, 1989) which may provide protection against gastrointestinal disorders including gastrointestinal infections, inflammatory bowel diseases and even cancer (Saarela et al., 2000). Additional health benefits atributed to probiotics are the stimulation of immune system, blood cholesterol reduction, enhancement of the digestibility of protein and vitamin synthesis, anti-bacterical activities, treatment of lactose intolerance and food-related allergies (Ouwehand et al., 2009; De Smet et al., 1998; Lee and Salminen, 2009; Lomer et al., 2008; Prescott and Bjorksten, 2007). Although the whole idea of probiotics is not new and their usage in treating body ailments is even mentioned in the Biblical scriptures, it was only in the early 20th century that the Russian scientist Elie Metchnikoff gave the first scientific explanation for the beneficial effects of lactic acid bacteria and proposed the concept of probiotics as it is known today (Ranadheera et al., 2010).
Originally, probiotic delivery was consistently associated with foods, particularly dairy foods, such as yoghurts and fermented milks. However, lactose intolerance, cholesterol content and allergenic milk proteins are the major drawbacks related to the intake of dairy products. Also, dietary habits, traditional and economic reasons limit the use of dairy products in some

countries (Granato et al., 2010). Furthermore, milk-based products must be maintained at refrigerated temperatures and are characterized by relatively short shelf-life. In this context, the development of new non-dairy probiotic foods is of great importance. An alternative strategy to increase the efficacy of a probiotic treatment would be to use a food matrix which naturally contains a higher content of ingredients with protective properties. Chocolate is one such product that meets many of these demands. Nowadays chocolate is not just an indulgent snack, it is also increasingly recognised as possessing many qualities that are beneficial to health. This is particulary true of dark chocolate made with more cocoa solids due to its content of the polyphenol components, acting as natural antioxidants. In view of the growing market deman for products that combine delight with health, chocolate represent a good opportunity to add further health benefits via the use of probiotic cultures. In addition, according to a recent study conducted at the University of Ghent (Belgium), supported by Barry Callebaut, one of the leading manufacturers of cocoa and chocolate products, over 80 % of probiotics in chocolate as a "carrier" safely pass through the stomach, compared to only 20 % when the "carriers" are milk/yogurt drinks. This also means that a smaller amount of food is needed to achieve therapeutic effects. Cocoa-butter provides protection for probiotic bacteria, so it is not necessary to store chocolates in a refrigerator, and shelf-life of the product by far exceeds that of dairy based foods (www.maramor.com).

However, incorporation of probiotic in food is linked to some serious obstacles arising from the definition of probiotics itself: they need to reach digestive tract, a place of their action, alive and in sufficient number. For probiotic functionality, the numbers of live cells of these bacteria in foods should not be lower than 10^6 cfu/g at the time of consumption (Patel et al., 2008).Their survival is linked to several factors: first the endogenous properties of the chosen bacteria strain, their environment (other ingredients properties, humidity, temperature, oxygen…), the digestive process (gastric acidity, biliary salts), as well as various mechanical stresses linked to food processing (Maillard and Landuyt, 2009). To overcome these limitations, it is necessary to apply new or adapt existing technological process to allow, not only the survival of conditions during production, but also sufficient viability of probiotic bacteria during extended storage. This represents a great challenge for technologists and manufacturers of non-dairy products, which are usually kept at room temperature.

Combination of enrichment of dark chocolate with viable cells of probiotic bacteria, together with other favorable components in its composition (poliphenols), would represent a new funtional product whose influence on the health of entire population is inasmuch greater given that chocolate is preferably eaten by children and teenagers. Because the unique taste of chocolate is particularly valuable for consumers, its sensory attributes should remain unaltered despite the addition of preparation of probiotic bacteria. Sensory properties of chocolate are among the most important parameters in defining its overall quality (Torres-Moreno et al., 2011). General sensory acceptability and appealing by consumers is the key factor for successfully placing chocolate on the market. A melted chocolate is a complex suspension of at least two dispersed phases (Beckett, 2009), but with addition of probiotic culture, a new solid phase is introduced. This can affect the crystallisation of cocoa butter in chocolate and lead to appearance of fat bloom, and/or changes in structural properties and occurrence of difficulty in chewing. Also, textural changes can cause differences in release of volatile aroma compounds in the mouth headspace, thus influencing the perception of both odor and taste of chocolate (Afoakwa et al., 2009). For these reasons, the significance of sensory analysis in the process of development of new products with funtional properties is crucial.

Sensory attributes of dark chocolates supplemented with probiotic strain *Lactobacillus acidophilus* NCFM® and survival of these bacteria during storage of 180 days at different temperatures were examined within the scope of presented work. Production of this sort of chocolate is a novel approach in the area of successful applications of lactic acid bacteria, including their probiotic strains, for manufacturing of non-dairy products.

MATERIAL AND METHODS

Probiotic preparation

Dark chocolate mass, that is dark chocolates supplemented with live cells of a *Lactobacillus* strain with probiotic properties were the examined material.

Concentrated probiotic preparation in lyophilized form, which contained probiotic strain *Lactobacillus acidophilus* NCFM® , was used. This strain was obtained from Danisco (Denmark), and is commercially known as HOWARU DOPHILUS. Declared starting cell count in the preparation was ≥ 10^{11} DCU.

Chocolate production

Dark chocolates composition was as follows: sugar (sucrose), cocoa mass, cocoa butter, emulsifier (soy lecithin), flavour (ethylvanillin), and they contained 44 % of cocoa solids. Chocolate production was carried out in industrial conditions in the joint-stock company „Štark“ (Belgrade, Serbia). To obtain chocolate mass, production was done folowing the standard technological operations, with one alteration upon completion of tempering. Namely, cocoa mass, part of cocoa butter, sucrose and 1/3 of emulsifier were mixed in a mixer for 2 hours. Obtained mass was subjected to coarse, and then fine grinding (refining) on the five – rollers machine, until an average size of solid particles reached approximately 25 μm. Subsequently, after adding the remaining cocoa butter and 2/3 of lecithin (in two portions), the conching operation was performed at 75°C during 9 hours. Tempering was carried out in multistage heat exchanger with strict control of temperature in order to gain appropriate form of cocoa fat crystals (β V) (cooling to ~ 28°C and rise up to 32°C). After tempering, dark chocolate mass was separeted from the production line with purpose of dispersion of probiotic preparation in the amount of 3.5g/kg. This was done for optimizing stability of live probiotic cells by introducing them at lower temperatures. Next, chocolate mass containing probiotic bacteria was poured to forms, and then returned to production line for cooling, and removing from the forms. Finished chocolates were packed in lacquered aluminium foil, and cardboard checks on the packing machine.

Microbiological analysis

Lactobacillus acidophilus NCFM® was determined on agar MR-IM medium supplemented with 20 % maltose solution, incubated at 37˚C for 48 hours in the facultative anaerobic conditions in a Gas-Pak system (BBL, Germany). All measurements were performed in triplicate. Live cells were enumerated as colony forming units (cfu). Survival of probiotic bacteria cells was determined immediately after production and after 90 and 180 days of storage. In order to appoint the recommended storage temperature suitable for this kind of products, samples designed for microbiological analysis were keept at 4°C and 20°C and analyzed at the indicated periods.

Sensory analysis

The overall sensory quality of dark chocolate samples with probiotic bacteria during storage was determined using modified scoring method of sensory evaluation of chocolate (Popov-Raljić, Laličić-Petronijević, 2009).

Sensory evaluations included the selected, representative, or dominant attributes of chocolate quality, i.e.: appearance (color, gloss, form and surface); texture (structure, snap, hardness, chewiness); aroma (odor, taste). During evaluating the scoring range from 1.00 to 5.00, with the possibility of assigning half and quarter points, was applied. Nonetheless, Planck (Joksimović, 1977) as far back as 1947, introduced and scientifically explained the so-called “weight coefficient”. With application of the weight coefficients, a quantitative expression of the total product quality is obtained as the “weighted” mean value of the scores for each evaluated parameter. Because of that, prior to performing the evaluation, it is important to determine weight coefficient for each property, and balance them in such a way, that their sum equals to 20. Summarizing the individual scores, a complex indicator representing overall sensory quality of chocolates, is obtained. This indicator is expressed as

"% of the maximum possible quality". Dividing this value by the sum of weight coefficient, a weighted average value of ratings is gained, which also represents the overall sensory quality of chocolate. Quality category was determined in dependence of scores spans; samples which were evaluated with less than 2.5 points were considered as unsatisfactory, i.e. as inacceptable; scores within limits 2.5 – 3.5 characterized good quality products, 3.5 – 4.5 very good quality and 4.5 – 5 – excelent products.
Sensory evaluation of dark chocolates with probiotic bacteria was performed by five experienced assessors. Panelists all met the criteria specified by the ISO standards for selection, training and monitoring of assessors (ISO 8586-1, 1993; ISO 8586-2, 1994).

Statistical analysis
Basic parameters of the descriptive statistics included calculations of the arithmetic mean values (X_m), and variability parameters of the investigated sensory properties of dark chocolate samples supplemented with probiotic bactera, included determinations of standard deviations (S_d) and variation coefficients (C_v) expressed in percents (Hadživuković, 1991).

RESULTS AND DISCUSSION

The results of survival of incorporated probiotic strain *Lactobacillus acidophilus* NCFM® in dark chocolate are shown in Figure 1.

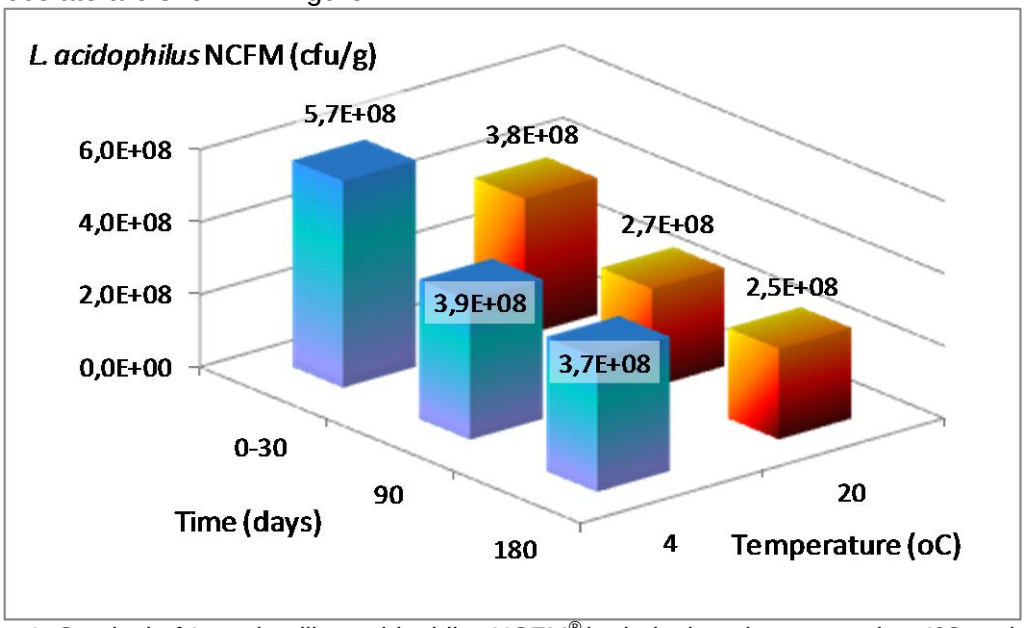

Figure 1. Survival of *Lactobacillus acidophilus* NCFM® in dark chocolates stored at 4ºC and 20ºC during period of 180 days.

As it can be seen from the results, probiotic strain *Lactobacillus acidophilus* NCFM® exibited a very good viability in dark chocolates with 44 % of cocoa solids. Although a slightly higher number of cells survived at a lower tempareture, it can be concluded that during the entire storage period of 180 days the number of viable cells remained at the same level of 8 log cfu/g at the refrigerator temperature (4ºC) as well as room temperature (20ºC). Despite a gradual decrease in a number of live probiotic cells with time, their count was maintained at the functional level. Among many factors affecting the survival of probiotic bacteria in confectionery, water activity, osmotic tension, oxygen and temperature could be considered as critical (Mattila-Sandholm et al., 2002).In this respect, a first step to protect the viability of probiotic bacteria represented their incorporation in dark chocolate mass at fairly low temperature during the manufacturing process (at ~ 30ºC). Furthermore, inside the chocolate bars the penetration of oxygen is very limited, so its harmful impact on probiotic bacteria is minimized due to the virtually anaerobic environment. Besides, chocolate is a product with very low water activity and with high concentration of fats, all of which favorably

affect the survival of probiotic bacteria. Package of chocolates in a double packaging material additionaly protect LAB during storage. All of the above contributed to a satisfactory survival of *Lactobacillus acidophilus* NCFM® in the dark chocolates during storage at 4ºC and 20ºC for up to 180 days. Obtained results are in agreement with findings of other authors (Nebesny et al., 2007; Zyzelewicz et al., 2010).
Addition of lyophilized preparation of *Lactobacillus acidophilus* NCFM® did not change the sensory properties of dark chocolates, as follows from the results of sensory analysis shown in Table 1.
Evaluated samples has high scores for almost every sensory attribute which were retained throughout the experiment. Distinguished gloss of tested samples indicated that there was no appearance of fat bloom, despite adding of new solid phase to the dark chocolate system. Samples were characterized by an appropriate structure and hardness, shell-like snap and good chewiness. Most importantly, the addition of probiotic bacteria did not disrupt the odor and taste of dark chocolate samples and these attributes were given excellent marks by the members of the panel. Based on weighted average value dark chocolate samples enriched with *Lactobacillus acidophilus* NCFM® belonged to the category of excellent sensory quality in all testing periods during storage of 180 days.

Table 1. The results of sensory analysis of dark chocolates supplemented with probiotic cells during storage of 180 days

Time of storage (days)	Calculated indicators	Evaluated sensory properties					Percent of the maximum possible quality	Weighted average value
		APPEARANCE	TEXTURE		AROMA			
		Visualy evaluated color, gloss, form and surface	Visualy evaluated structure and snap, palpatory evaluated hardness	Orally evaluated chewiness	Olfactory evaluated odor	Orally evaluated taste		
		WEIGHT COEFFICIENT						
		2.00	3.00	4.00	4.00	7.00		
0-30	X_m	9.70	14.25	19.80	20.00	35.00	98.75	4.94
	S_d	0.22	0.18	0.11	0.00	0.00		
	C_v	4.61	3.72	2.26	0.00	0.00		
90	X_{sr}	9.40	13.95	19.60	20.00	34.65	97.60	4.88
	S_d	0.11	0.14	0.14	0.00	0.11		
	C_v	2.38	2.94	2.79	0.00	2.26		
180	X_{sr}	8.90	12.30	19.00	19.80	33.95	93.95	4.70
	S_d	0.11	0.22	0.18	0.11	0.11		
	C_v	2.51	5.45	3.72	2.26	2.82		

CONCLUSIONS

Probiotics can be applied to dark chocolates with good efficiency. As the lipids in chocolate provide a nice barrier to the environmental conditions, the stability of probiotics in chocolate can be kept quite high compared to other applications. Also, dark chocolates containing probiotic bacteria have extended shelf-life in relation to dairy products. Although, when stored at 4ºC slightly higher number of viable probiotic cells is achieved, the results of our study show that the chocolates with probiotic bacteria may be stored at room temperature as well, without a significant decrease in cell viability. Enrichment with probiotic strain *Lactobacillus acidophilus* NCFM® did not change the sensory attributes of dark chocolates. Therefore, dark chocolate represent a good matrix for probiotic delivery, and at the same time, because of added value, contributes to expanding assortment of both confectionery products and functional foods.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project 046010 “The development of new encapsulation and enzymatic technologies for production of biological active substances and other food components in order to increase competitiveness, quality and safety of the products”, financed by the Ministry of Science and Technological Development, Republic of Serbia.The authors want to thank the joint-stock company „Štark“ (Belgrade, Serbia) for providing raw materials, facilities and personnel to perform this experiment.

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DETERMINATION OF FATTY ACIDS DURING MILK FERMENTATION BY KOMBUCHA CULTIVATED ON BLACK TEA

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ABSTRACT: This study evaluated the impact of the different fermentation temperatures on the fatty acids profile of fermented milk beverages produced by kombucha cultivated on black tea.
Milk with 2 g/100g was inoculated with 10 mL/100mL kombucha inoculum. Fermentations were performed at 37°C and 42°C until pH 4.60 was reached. Content of fatty acids was determinated during fermentation on pH 6.07; 5.10 and 4.60. Analyses of fatty acids composition were carried out by gas chromatography – mass spectrometry.
The results of this investigation indicate that fatty acids profile were similar in milk and fermented milk beverages produced by kombucha. The level of palmitic acid was the highest of all fatty acids among investigated samples.
Key words: *fermented milk beverages, kombucha, fatty acids*

INTRODUCTION

During the yoghurt production the microorganisms of the starter cultures play an important role in development of physicochemical, textural and sensory characteristics of the product. Nowdays, traditional process has been improved, and the basic changes depend on the following: the purity of commercially manufactured starter cultures; direct-to-vat inoculation (DVI) of starter cultures is widely used; incubation temperature can be accuratelly controlled so the rate of acid development and processing time is known in advance; cooling of the product can be carried out quickly at the desirable level of acidity resulting in uniform yoghurt quality; measuring the rate of acid development in milk during fermentation enables even a semi-skilled operator to control the process adequately.
Fat content of yoghurt differs from one country to another according to the existing or proposed standards, or alternatively in relation to the types of products. Although much quantity of fermented dairy products sold worldwide is manufactured from skimmed milk, traditional yoghurts have always contained around 3-4 g/100g milk fat, thus concentrated yoghurt (labneh) or Greek-style yoghurt contain 9-10 g/100g (Tamime and Robinson, 2004). The influence of milk fat content on yoghurt properties, especially the consistency and flavour, is of great importance as well as the fact that lipids are an integral part of a balanced diet (Lori et al., 2000).
In the milk fat, triacylglycerols are the major lipid class accounting for 97-98 g/100g of the total lipids and the remaining fraction consists of phospholipids, sterols, fat soluble vitamins (A,D, E and K), fatty acids, waxes and squalene (Fox, 1994). It is important to stress that milk fat contains an extremely wide range of fatty acids. Most of these are present in the form of various glycerides, but over 400 individual fatty acids have been identified in cow's milk. The milk fats are characterized by the presence of relatively high concentrations of short-chain fatty acids, especially butiric and hexanoic acids. Appreciable amounts of medium-chain-length fatty acids are also present, but relatively low proportion of polyunsaturated fatty acids i.e. essential linoleic fatty acid (Fox, 1994).

The triacylglycerol lipase enzymes that can cause the enzymatic hydrolysis of milk fat in yoghurt may originate from starter culture or from microbial contaminants that survived the heat treatment of milk. Lipases which occur naturally in milk, are inactivated at ordinary pasteurization temperatures. Therefore, any reduction in the percentage of fat, or increase in the level of fatty acids (free or esterified) or volatile fatty acids content in yoghurt can be attributed to lipid metabolism by microorganisms of the applied starter cultures.
Hydrolysis of milk fat by starter cultures occures only to a limited degree. Formisiano et al. (1974) estimated decrease of 3.4 g/100g of milk fat in yoghurt stored for 21 days at 40°C. Fermentation of full fat milk with *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* or *L. acidophilus* resulted in different effects on lipids: significant increase in saturated fatty acids and oleic acid and a concomitant decrease in linoleic and linolenic acids in the glyceride fraction (Rao and Reddy, 1984). The total level of volatile fatty acids (VFA) increase during the manufacture and storage of yoghurt. The increase in the level of VFA is dependent of the starter bacteria strains, type of milk, duration and temperature of incubation, temperature of milk heat treatment and/or yoghurt shelf life (Tamime and Robinson, 2004).
During fermentation of milk, some strains of bacteria, as the result of their growth and metabolism, are able to change the fatty acid profile of milk and produce functional fatty acids (Yadav et al., 2007; Ekinci et al., 2008). Coskun and Ondul (2004) reported that the addition of mesophilic lactic acid bacteria to milk may contribute to the production of free fatty acids by spontaneous lipolysis during cold storage.
The aim of this research was to evaluated the impact of the different fermentation temperatures on the fatty acids profile of fermented milk beverages produced by kombucha cultivated on black tea.

MATERIAL AND METHODS

Milk

Homogenized and pasteurized milk from AD Imlek, Division Novi Sad Dairy, was used for the production of kombucha fermented milk beverage. The composition of milk was as follows: fat content – 2.00 g/100g, total solids – 10.59 g/100g, total proteins – 3.30 g/100g, lactose – 4.60 g/100g and ash – 0.69 g/100g.

Kombucha inoculum

Kombucha inoculum was prepared according to procedure published by Malbaša et al., (2009). Kombucha is cultivated on black tea (*Camellia sinensis* – oxidized, 1.5 g/L) with saccharose concentration of 70 g/L. The tea was cooled to room temperature, after which inoculum from a previous fermentation was added in concentration of 10%. Incubation was performed at 25°C for 7 days. Inoculum in concentration of 10 mL/100mL was used for milk inoculation.

Samples production

Samples were produced by addition of 30 mL of kombucha inoculum in 300 mL of milk at two different temperatures: 37°C and 42°C simultaneously. Fermentation was continued until pH= 4.6 was reached. Samples were taken at pH values: 6.07, 5.10 and 4.60 and cooled to 4°C.

Determination of fatty acids in samples

Fat was extracted from samples (4 cm^3) by adding methanol (4 cm^3) and chloroform (4 cm^3). The mixture was shaken vigorously for 1 min. and then centrifuged at 3000 rpm for 10 min. under room temperature. The lower phase containing the lipid fraction was isolated and evaporated to dryness under nitrogen (Havemose et al., 2004).
Previosly extracted fats were dissolved in hexane (2.4 cm^3). An aliquot (0.6) of 2 mol/dm methanolic KOH solution was added. The tube was capped and vigorously shaked for 20 s and allowed to boil 1min. in water bath at 70°C. After 20 s of shaking 1.2 cm^3 of 1 mol/dm HCl was

added and gently stirred. After phase separation the upper phase containing the fatty acid methyl esters was decanted and 1µL was used for further analysis (Kravić et al., 2011).
The analysis of fatty acid methyl esters were performed on a Hewlett–Packard (HP) 5890 gas chromatograph coupled with a HP 5971A mass spectrometer detector. Supelco fused silica capillary column SP–2560 (100 m x 0.25 mm i.d., 0.20 µm) was used. The inlet temperature was 230°C with a split ratio 1:40, and the carrier gas was helium with constant flow rate of 0.58 cm^3/min. The initial temperature of 100°C was held for 5 min. and increased by 6°C/min to an end temperature of 240°C. The mass spectrometer was operated in the electron ionization mode with quadruple temperature of 180°C. Data acquisition was carried out in the scan mode (range 50–400 m/z).

RESULTS AND DISCUSSIONS

The fatty acids content in milk and samples during fermentation on 37°C and 42°C are shown in Table 1. The level of palmitic acid (C16:0) in milk and samples was the highest of all fatty acids. In milk that was 27.31 g/100g of total fatty acids, in fermented milk beverages on 37°C and 42°C (pH=4.60) 24.69 g/100g and 25.07 g/100g, respectively. Beside the palmitic acid three fatty acids (C18:1, C14:0 and C18:0) were found to be predominant acids in milk and all samples. They accounted 45.27 g/100g (milk), 46.61 g/100g (fermented milk beverages on 37°C and pH=4.60) and 49.52 g/100g (fermented milk beverages on 42°C and pH=4.60) of total fatty acids.
There is no significant difference between samples in fatty acids content during fermentation on 37°C and 42°C. The content of myristic acid (C14:0) in samples produced on 37°C was 12.02 g/100g (pH=6.07), 12.68 g/100g (pH=5.10) and 12.37 g/100g (pH=4.60) while in samples produced on 42°C was 11.52 g/100g (pH=6.07), 11.41 g/100g (pH=5.10) and 11.57 g/100g (pH=4.60).
Milk fat typically contains a high proportion of saturated fatty acids (SFA; 70–75 g/100g) and monounsaturated fatty acids (MUFA; 20–25 g/100g) and small amounts of polyunsaturated fatty acids (PUFA; 5 g/100g) (Lock and Shingfield, 2004). Obtained results are in accordance with that. The medium–chain SFA (*i.e.*, C12:0, C14:0 and C16:0) which account for the majority of SFA in milk fat have been implicated in increasing total and low density lipoprotein (LDL) cholesterol concentrations (Williams, 2000). Stearic acid (C18:0) is considered to be neutral in this regard. Generally, PUFA and MUFA are regarded as beneficial to human health. In this research milk showed the highest content of SFA. However, fermented milk beverages produced on 37°C and 42°C (pH=4.60) had higher content of MUFA, as well as PUFA compared with milk.

Table 1. Fatty acids content (g/100g of total fatty acids; means±SD (n=3)) in milk and samples during fermentation on 37°C and 42°C

Fatty acid content	Milk	Samples					
		pH=6.07		pH=5.10		pH=4.60	
		37°C	42°C	37°C	42°C	37°C	42°C
C4:0	2.05±0.19	1.96±0.20	1.56±0.14	2.15±0.28	1.90±0.15	2.11±0.30	1.90±0.27
C6:0	1.81±0.13	1.57±0.17	1.23±0.17	1.51±0.19	1.31±0.09	1.54±0.21	1.29±0.16
C8:0	1.19±0.14	1.10±0.03	0.73±0.06	1.03±0.32	0.70±0.13	0.97±0.09	0.72±0.09
C10:0	3.06±0.27	2.60±0.30	2.21±0.14	2.81±0.32	2.09±0.21	2.68±0.30	2.23±0.18
C12:0	4.16±0.33	3.45±0.14	3.05±0.13	3.46±0.16	2.78±0.15	3.51±0.13	2.91±0.11
C14:0	13.95±0.33	12.02±0.43	11.52±0.36	12.68±0.41	11.41±0.21	12.37±0.17	11.57±0.41
C15:0i	0.21±0.03	0.23±0.03	0.29±0.03	0.24±0.03	0.25±0.03	0.30±0.04	0.25±0.04
C15:0a	0.54±0.07	0.54±0.07	0.60±0.07	0.53±0.02	0.54±0.07	0.60±0.04	0.50±0.05
C14:1	1.28±0.13	1.14±0.08	1.06±0.08	1.10±0.05	0.91±0.09	1.19±0.08	0.90±0.04
C15:0	2.06±0.16	2.00±0.18	2.03±0.17	2.04±0.08	1.88±0.24	2.17±0.16	1.82±0.09
C16:0i	0.39±0.04	0.40±0.04	0.52±0.07	0.40±0.04	0.45±0.05	0.49±0.06	0.41±0.06
C16:0	27.31±0.52	24.51±0.43	23.87±0.88	25.09±0.54	25.41±0.64	24.69±0.35	25.07±0.74
C17:0i	0.50±0.07	0.55±0.06	0.61±0.08	0.59±0.09	0.61±0.09	0.66±0.09	0.58±0.09
C16:1	3.32±0.22	3.53±0.25	3.52±0.23	3.59±0.10	3.20±0.25	3.72±0.20	3.34±0.16
C17:0	1.29±0.06	1.36±0.12	1.39±0.16	1.42±0.15	1.33±0.15	1.57±0.19	1.38±0.14
C17:1	0.45±0.06	0.53±0.06	0.51±0.06	0.52±0.07	0.43±0.07	0.52±0.05	0.41±0.07
C18:0	10.75±0.56	12.97±0.21	14.04±0.22	12.61±0.47	13.84±0.16	12.80±0.20	13.80±0.16
C18:1t	1.73±0.13	2.01±0.12	2.47±0.09	1.96±0.14	2.19±0.13	1.98±0.16	2.25±0.25
C18:1c	18.84±0.81	20.26±0.26	21.48±0.26	19.79±0.29	21.89±0.21	19.46±0.37	21.90±0.39
C18:2c	3.51±0.20	4.82±0.23	4.37±0.30	4.34±0.22	4.35±0.40	4.28±0.24	4.35±0.25
C20:0	0.32±0.05	0.46±0.06	0.34±0.03	0.35±0.03	0.32±0.04	0.34±0.06	0.35±0.05
C20:1	0.32±0.04	0.39±0.05	0.52±0.08	0.42±0.06	0.41±0.08	0.46±0.06	0.40±0.06
C20:2	0.95±0.12	1.58±0.12	2.09±0.27	1.38±0.19	1.81±0.18	1.59±0.16	1.66±0.25
SFA	69.60	65.74	63.99	66.91	64.81	66.81	64.78
MUFA	25.94	27.85	29.55	27.38	29.03	27.32	29.21
PUFA	4.46	6.41	6.46	5.71	6.16	5.86	6.02

SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids

CONCLUSION

The present study examined the changes in fatty acids content during fermentation on two different temperatures (37°C and 42°C) of fermented milk beverages produced by kombucha cultivated on black tea. Result showed that there is no significant difference between samples in fatty acids content during fermentation. Also, fermented milk beverages produced on 37°C and 42°C (pH=4.60) had higher content of MUFA, as well as PUFA compared with milk. That increases must be attributed to the kombucha. It is therefore possible to obtain fermented milk beverages by kombucha with potential health benefit.

ACKNOWLEDGMENT

Financial support from the Ministry of Education and Science of the Republic of Serbia (Grant III 46009) is highly appreciated.

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EFFECT OF ENCAPSULATED AUTOCHTONOUS POTENTIAL PROBIOTIC BACTERIA *LACTOBACILLUS PARACASEI* 08 ON THE CHARACTERISTICS OF THE SOFT GOAT CHEESE

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ABSTRACT: The survival of the encapsulated potential probiotic strain *Lactobacillus paracasei* 08 and commercial probiotic bacteria *Lactobacillus acidophilus* LA-5 and their effect on the soft goat cheese production were investigate**.** Three batches of soft goat cheeses were produced using mesophilic lactococci starter culture CHN 11 (*Lactococcus lactis* and *Lactococcus cremoris,* Chr. Hansen): 1. control cheese (starter); 2. starter + spray-dried potential probiotic *Lb. paracasei* 08 (Culture Collection of the Department for Food Microbiology, University of Belgrade); 3. starter + freeze-dried commercial probiotic *Lb. acidophilus* LA-5 (Chr. Hansen).
Cheese samples were assessed for viability of encapsulated probiotic bacteria, starter bacteria counts, chemical composition, pH values and sensory evaluation during 5 weeks of storage. *Lb. paracasei* 08 and *Lb. acidophilus* LA-5 counts maintained at the level of 10^7-10^8 cfug^{-1}. Starter bacteria counts were >10^6 cfug^{-1} in all cheeses samples. The chemical composition and pH values of probiotic cheeses haven't been significantly different from the control cheese. Sensory evaluation has shown that cheese produced with *Lb. acidophilus* LA-5 had an acceptable sensory quality, while control cheese and cheese produced with *Lb. paracasei* 08 had a high sensory quality.
The data demonstrate that the spray-dried potential probiotic strain *Lb. paracasei* 08 could be successfully used in the soft goat cheeses production, without significant effect on the cheese quality during storage.
Key words: *encapsulated probiotic, soft goat cheese, chemical composition, sensory properties*

INTRODUCTION

Food that contains probiotic bacteria belongs to the functional food category that claimed to have positive effect on human health beyond their contribution to basic nutrition (Roberfroid, 1998). Studies have shown that functional foods with probiotic bacteria have a beneficial influence on human health by improving the balance of intestinal microbiota and mucosal defenses against pathogens (Cínthia and Susana, 2009).
The survival of probiotic bacteria could be preserved with microencapsulation method spray-drying, which is most commonly used method in the food industry. The process of spray drying involves the dispersion of the core material into a polymer solution, forming an emulsion or dispersion, followed by homogenization of the liquid, then atomization of the mixture into the drying chamber (Jackson and Lee, 1991). Spray drying is desirable for commercial application, enabling convenient transport and storage of cultures and their application in functional food (Gardiner et al., 2002). It is economical, easily scaled up and uses equipment readily available in the food industry (Gibbs et al., 1999). However, cell injury emerges during heat stress and spray drying, which can potentially destroy some of the characteristics of the probiotic bacteria strains (Teixeira et al., 1997; Gardiner et al., 2000).
The nutritional and health benefits of goat milk are related to a number of medical problems of people foremost being food allergies with cow's milk proteins (Walker, 1964). Fermented dairy products, such as yogurts, fermented milk and cheeses are good delivery system for

probiotic bacteria. Nevertheless, cheese have higher pH, higher fat content, buffering capacity and solid matrix, so they protect probiotic bacteria more efficiently during the storage of the food and its passage thought the gastrointestinal tract (Kailasapathy and Chin, 2000; Vinderola et al., 2002). For these and many other reasons, there are numerous studies on various types of cheeses with probiotic bacteria, such as Cheddar cheese (Phillips et al., 2006), Turkish white cheese (Kasimoğlu et al., 2004), Cottage cheese (Heller, 2001), Caprine cheese (Gomes et al., 1998).
There is limited knowledge about soft cheese made from goat's milk with spray-dried probiotic bacterial strains. Therefore, the objective of this study was to determine viability of survival of potential spray-dried probiotic bacteria and commercial probiotic bacteria in soft cheeses made from goat milk during 56 days of storage, and their effect on chemical properties and sensory quality of cheeses.

MATERIAL AND METHODS

Probiotic bacteria strains

Potential probiotic strain used in this study *Lb. paracasei* 08, was isolated from traditionally made white brined cheese (Radulović, 2007). *Lb. paracasei* 08 was selected taking into account its technological and probiotic potential properties which are tested by Radulović et al. (2010, 2011). Commercial probiotic strains used in study is lyophilized *Lb. acidophilus* LA-5 (Chr Hansen, Denmark).

Spray-drying conditions

Spray-drying test were performed using the method of Petrović (2011). Overnight culture of *Lb. paracasei* 08 (300ml) was centrifuged (4500 x g, 15min, 15˚C). The pellet was washed twice in 50mM K_2HPO_4 (pH 6.5) and resuspended in 300ml of the sterile reconstituted skim milk (20% w/v). Inoculated sterile reconstituted skim milk was spray-dried with a laboratoryscale spray-dryer (model B-290 Buchi mini spray dryer, Switzerland), using a constant inlet air temperature of 170˚C and outlet temperature 80˚C.

Cheese production

Milk was pasteurized at 85°C for 10 min. After the milk was cooled to 30°C, it was divided into three equal parts. Control batch (C1) was produced using commercial starter culture mix CHN 11 *Lactococcus lactis* and *Lactococcus cremoris* (Chr. Hansen, Denmark). Test batches C2 contained starter culture CHN 11 + spray-dried potential probiotic strain *Lb. paracasei* 08, while test batch C3 contained starter culture CHN 11 + commercial probiotic strain *Lb. acidophilus* LA-5. Each batch was inoculated with starter cultures (1mL/100mL), after which potential and commercial probiotic strains were added in order to reach 7 log $cfug^{-1}$. After the cultures addition, solution of rennet (Sacco Clarifici, Italia) was added in concentration of 0.5g/100 L^{-1} to each batch. Fermentation lasted for 15 hours. After fermentation, cheeses were cut into small cubes, placed in sterilized cotton cheese cloth, and allowed to drain for 10 hours at 15°C. After draining, the cheeses were salted with 1% of NaCl and packed in individual plastic cups. Cheeses were stored under refrigeration temperature at 4°C for 56 days. Sampling was carried out on storage days 1, 7, 21, 35 and 56 day for microbiology analysis and on 7 and 21 day for chemical and sensory analysis.

Microbiological analysis

From each cheese variant, 10 g of cheese were weighed aseptically and transferred into an sterile Stomacher bag under aseptic conditions and homogenized in 90 ml of sterile sodium citrate 2% (w/v) for 5 minutes using Lab Blender 400 stomacher (Seward, London, UK). Appropriate decimal dilutions of the samples were prepared using the same diluents and plated on different growth media. Starter bacteria counts were determined on M17 agar (Oxoid, CM 785) aerobically at 30°C for 48 h. Potential probiotic strain *Lb. paracasei* 08 was enumerated on MRS agar (Oxoid, CM 361) and incubated anaerobically (Gas Pak, BBL,

Germany) for 48 h on 37°C. Colonies of this strain was different from NSLAB as specific mucoid colonies which were EPS positive. This characteristic was determined in previously studies (unpublished data). Commercial probiotic strain *Lb. acidophilus* was enumerated on MRSiM agar with addition of maltose dilution (200g/l) and incubated anaerobically (Gas Pak, BBL, Germany) on 37°C for 48 h. Microbiological data were transformed into logarithms of the number of colony-forming units ($cfug^{-1}$).

Chemical analysis

Cheese samples were analyzed for the determination of total solid content according to the IDF standard method (1982), fat content according to Van Gulik butyrometers methods (IDF, 1986) and determination of nitrogen content were analyzed according to the IDF standard method (2002). The pH value of cheese slurry was measured by a pH meter (Consort, Belgium).

Sensory analysis

Sensory analysis of all variants of chesses, were conducted after 7 and 21 days of storage. Five panel members, selected and trained for sensory analysis on Faculty of Agriculture, University of Belgrade, evaluated cheese for exterior and interior appearance, body and texture, and flavour (odour and taste) using 5-point scale, with 1 being the worst and 5 the best quality. Depending on the importance of attributes, they were multiplied by 3, 7 and 10, respectively. The total sensory quality (100) was expressed as a percentage of the maximum quality.

RESULTS AND DISCUSSION

The viability of potential probiotic *Lb. paracasei* 08 and commercial probiotic *Lb. acidophilus* LA-5 are shown in Figure 1.

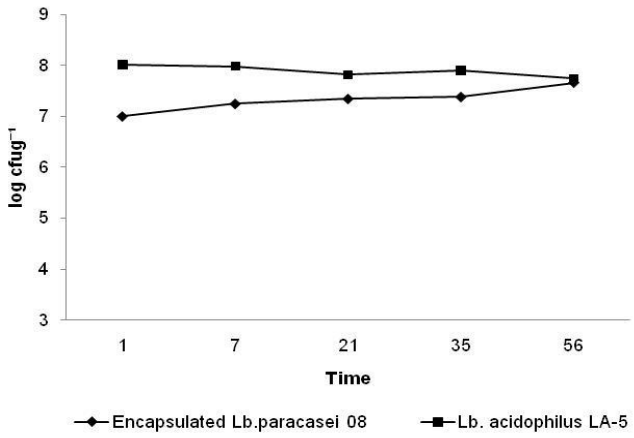

Figure 1. Survival of potential spray-dried and commercial probiotic bacteria during storage

At the first day of storage, potential spray-dried probiotic strain *Lb. paracasei* 08 count was 7 log cfu g^{-1} and after 56 days it was 7.69 log cfu g^{-1}. *Lb. acidophilus* LA-5 count was 8.01 log cfu g^{-1} in the first day of storage, and 7.04 log cfu g^{-1} after 56 days of storage. It is recommended that the minimal concentration of probiotic in food should be 10^7 cfu/g at the moment of intake to assure a favorable impact on a consumer's helath (De Vuyst, 2000). Furthermore, these results are in accordance with studies by Gardiner et al. (2002), who added spray-dried *Lactobacillus paracasei* NFBC 338 in Cheddar cheese.

The number of starter lactococci in cheeses during storage is shown in Figure 2. There is no significant difference between C1, C2 and C3 cheeses for starter lactococci count during storage period.

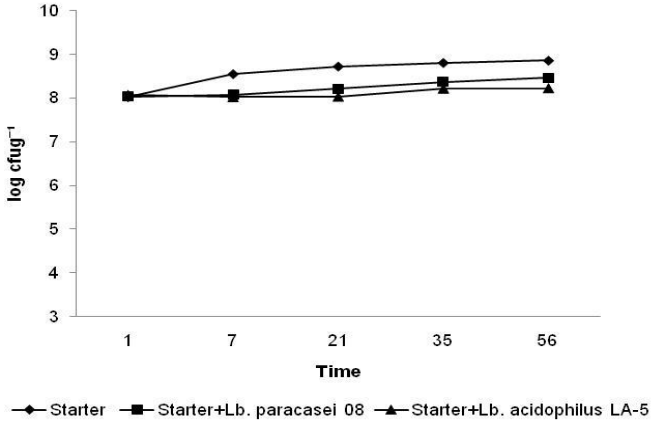

Figure 2. Starter bacteria count in cheeses during storage

Changes in chemical composition of cheeses are shown in Table 1.

Table 1. Chemical composition of cheeses during storage

Parameters	Dry matter (%)	Fat (%)	Protein (%)
Day 7			
Cheese C1	25.37	10.50	7.76
Cheese C2	24.25	9.00	7.50
Cheese C3	24.89	9.50	7.75
Day 21			
Cheese C1	25.24	12.00	7.03
Cheese C2	24.24	12.00	7.80
Cheese C3	24.89	12.00	7.65

After 7 day of storage, all variants of cheeses had similar dry mater content (24-25%). At the beginning of storage, there were some differences in fat content, but after 21 day of storage, all variants of cheeses had the same fat content (12%). Protein content in all variants of chesses was similar after 7 day of storage, but after 21 day of storage cheese C1 had some lower (7.03%), while cheeses C2 and C3 had higher protein content (7.80% and 7.65%).

Changes in pH values are shown in figure 3.

Initially pH ranged from 4.08 and 4.09 for cheeses C1 and C2, and 4.16 for cheese C3. At the end of storage, cheese variants reached their minimum pH values of 3.75-3.81. There is no significance difference in chemical composition between control cheese with starter culture (C1) and cheeses with added probiotic strains (C2 and C3).

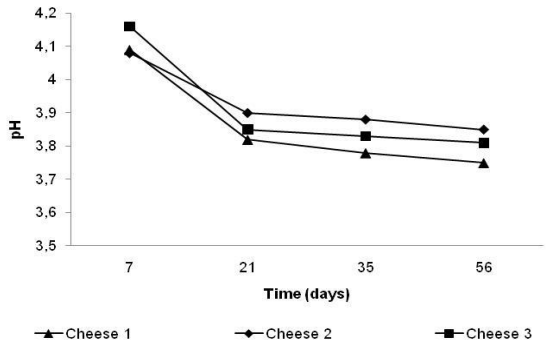

Figure 3. Changes in pH values of cheese during storage

Table 2. The sensory evaluation of cheeses

Sensory attributes*		Exterior and Interior Appearance		Body and texture		Flavor and odour		% Maximum quality
Cheeses	Time (days)	R*	W*	R*	W*	R*	W*	
Cheese 1	7	4.9	14.70	4.9	34.30	4.5	45.00	94.00
	21	5.0	15.00	5.0	35.00	4.2	42.00	92.00
Cheese 2	7	4.88	14,63	5,00	35,00	5,00	50,00	99,63
	21	5.00	15.00	4,80	33,60	4,10	41,00	89,60
Cheese 3	7	4.88	14.64	4.88	34.16	3.75	37.50	86.30
	21	5.0	15.00	4.80	33.60	2.8	28.00	76.60

**R-rating (mean value); W-weighted (mean value multiplied with coefficient): Appearance 3; Body and texture 7; Flavour and odour 10*

The results of sensory analysis are shown in table 2. Cheese samples were evaluated after 7 and 21 day of storage.
After 7 day of storage, all cheese samples were grated high for every parameter of sensor quality. However, cheese C3 with commercial probiotic *Lb. acidophilus* LA-5, received lower grades for flavour. After 21 day of storage, all variants of cheeses were similar grated for exterior and interior appearance and for body and texture, but there were some difference in flavour grades. Cheese C2 with potential spray-dried probiotic *Lb. paracasei* 08 was similar graded as control variant, while cheese with commercial probiotic *Lb. acidophilus* LA-5 had the lowest grade for flavour (2.8). All variants were grated as acceptable.

CONCLUSIONS

The current study showed that spray-dried potential probiotic *Lb. paracasei* 08 and commercial probiotic *Lb. acidophilus* LA-5 can be successfully used as adjunct cultures in production of probiotic soft cheeses made from goat's milk. Probiotic strains maintained viability of > 7 log cfu g^{-1} during storage period. There were no significante differences in starter bacteria count. Chemical composition and pH values were similar between control cheese and probiotic cheeses. In sensory analysis both probiotic cheeses received high scores, but better aroma, taste and texture were detected in cheese with spray-dried potential probiotic bacteria *Lb. paracasei* 08. These results indicate that autochthonous potential spray-dried probiotic strain *Lb. paracasei* 08 is suitable for development of an acceptable probiotic soft goat cheeses as new functional product.

ACKNOWLEDGEMENTS

The authors of this paper highly appreciate a financial support from the Ministry of Education and Science of Republic of Serbia, National Project No. 046010 (2011-2014) and Bilateral Project, Republic of Serbia and Republic of Slovenia (2010-2011), BI-SL/10-11-035.

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EFFECT OF FERMENTATION BY *STREPTOMYCES* SP. ON ANTIOXIDANT PROPERTIES OF SPENT COFFEE EXTRACTS

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ABSTRACT: During espresso coffee preparation large amounts of spent coffee residues are produced and discarded daily as a part of the communal waste. However, there is a great potential in recycling this type of waste for extraction of natural antioxidants and manufacture of dietary supplements. The aim of this study was to investigate the effect of fermentation on the antioxidant properties of spent coffee extracts. Six series of spent espresso-type coffee samples were inoculated with selected natural strains of Streptomyces sp. The solid state-fermentation was carried out at 30°C five days. Aqueous extracts have been prepared and evaporated in the spray-dryer. The total polyphenol content was determined by Folin-Ciocalteu method and the antioxidant activity by two methods: inhibition DPPH (2,2-diphenyil-1-picrylhidrazyl) radicals and FRAP (Ferric Reducing Antioxidant Power). Inhibition of DPPH radicals was expressed as IC_{50} value. Results were compared to those of nonfermented control sample. Fermentation of spent coffee with these cultures have resulted in increased contents of polyphenols. Also, both methods have confirmed increased antioxidative activity in certain samples depending on the selected strains. It was therefore concluded that fermentation of spent coffee with *Streptomyces* sp. cultures have improved its antioxidative properties which presents a potential for its application in pharmaceutical and food industry.
Key words: *spent coffee, fermentation, Streptomyces sp., antioxidant activity, polyphenols*

INTRODUCTION

The coffee tree belongs to the family *Rubiaceae*. There are about 70 different coffee species, but only two of these are commercially explored worldwide: *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta) (Mussatto et al., 2011).
Coffee is one of the most popular beverages consumed by millions of people every day. Some studies have shown that consuming coffee has certain benefits on human health, so it manifests strong antioxidant and anti-tumor activity and limited anti-inflammatory and anti-allergic (Ramalakshmi et al., 2009). These properties are determined directly by the composition of coffee. Roasted coffee is composed by carbohydrates (38–42% dry basis), melanoidins (23%), lipids (11–17%), proteins (10%), minerals (4.5–4.7%), chlorogenic acid (2.7–3.1%), aliphatic acids (2.4–2.5%), caffeine (1.3–2.4%) (Esquivel and Jiménez, 2011). The beverage is very well known for the antioxidant properties of its components - polyphenols and phenolic acids, particularly chlorogenic acid, which is about 56 ±11% total polyphenols (Naidu et al., 2008).
During the production of consumer coffee a large amounts of waste are obtained. Due to the presence of organic material, spent coffee residues are highly pollutant residues, and represent a pollution hazard if discharged into the environment. Despite this negative characteristic, there are few studies focusing on their use in different and profitable applications. Many attempts have been made to use coffee waste as fertilizer, animal feed or as fuel (Saenger et al., 2001; Silva et al., 1998; Pandey et al., 2000).
Waste coffee, prepared from espresso coffee, is very appropriate for exploitation as raw material in the production of biologically valuable ingredients under solid-state fermentation (SSF) conditions. The most commonly used microorganisms for fermentation of coffee residue are filamentous fungi (Machado et al., 2012; Nava et al., 2006; Arellano-González et

al., 2011) but it is also reported the use of *Streptomyces* sp. (Orozco et al., 2008). *Streptomyces* are Gram-positive soil bacteria that are able to degrade many macromolecules such as proteins, cellulose, starch, lipids, and chitin. For the degradation of cellulose, hemicellulose, and lignin that are abundant in coffee, different strains of *Streptomyces* sp. have been studied and found to be good producers of cellulases (Jang and Chen, 2003).
The aim of this study was to investigate the effect of fermentation of selected natural strains of *Streptomyces* sp. on the antioxidant properties of spent coffee extracts for its possible application in pharmaceutical and food industry.

MATERIAL AND METHODS

Bacterial strains

Five isolates of *Streptomyces* sp. obtained from the forest (coniferous) land sample were selected for this investigation. All isolates showed a cellulolitic activity, as determinated by the method of staining iodine on CMC agar (Kasana et al., 2008) and morphological properties characteristic for the species *Streptomyces* (Kampfer,2006). By addition, three isolates (CKS 5, CKS 8 and CKS 18) produce extracellular, dark brown pigment, melanin. The strains were routinely grown on ISP1 broth medium (pancreatic digest of casein 5.0g/l; yeast extract 3.0 g/l) at 30 °C until sporulation occurred (5-7 days).

Substrate for fermentation

Spent coffee residues produced after espresso coffee ("Cream coffee" Italia) preparation were used as a substrate for fermentation. Spent coffee was dried in a thermostat at 44 ºC, 48 hours, until the moisture content of about 10%. 50 ml of distilled water was added into 300 ml Erlenmeyer flasks with 30 g (dry matter) prepared spent coffee and samples were sterilized in an autoclave, under standard conditions. After sterilization, prepared samples were cooled to room temperature and inoculated with 10 ml *Streptomyces* pellet suspension (previously grown 5 days at 30 ºC, with stirring, 150 rpm/min). All samples plus the control (without *Streptomyces*) were incubated at 30 ºC for 3 days.

Preparation of coffee extracts

After completing the period of fermentation, 100 ml of distilled water were added to samples and extraction was performed on a magnetic stirrer, at room temperature for 2 hours. After extraction, centrifugation was performed (10 minutes, 4500 rpm/min). Supernatant was separated and the residue was subjected to re-extraction, under the same conditions. The obtained supernatant was added to the above and a total volume of liquid extract was adjusted to 300 ml, to determine the total dry matter. Thus prepared extracts were stored in a refrigerator (4ºC).
Drying of prepared liquid extract was performed on the spray-dryer (Büchi). Spray drying conditions were: inlet (the default) temperature of 170 ºC and the outlet (the temperature of the sample) of about 80 ºC. The obtained powder samples were used for further analysis.

Determination of total polyphenols content

Total polyphenol content of powdered coffee extracts was determined using the Folin–Ciocalteu method (Djordjevic et al., 2009; Lee et al., 1998), with slight modifications. Powdered extracts were dissolved in distilled water at a concentration of 10 mg dry matter/ml. 500 μl of Folin – Ciocalteu´s reagent and 6 ml of distilled water were added in 100 μl appropriately dissolved extracts. After that, 2 ml of 15 % sodium carbonate solution was added and the whole solution was adjusted to 10 ml with distilled water. Absorbance was measured after 2 h at 750 nm in a spectrophotometer UV/VIS ultrospec 3300 pro (Amersham Bioscience). As a standard curve was used a solution of gallic acid and the results were expressed as mg of gallic acid (GA) per gram of dry matter extract.

DPPH radical scavenging activity

Free radical scavenging activity was measured by 1,1- diphenyl-2-picrylhydrazyl (DPPH) by method of Lee et al. (1998). Powdered coffee extracts were dissolved in distilled water at certain concentrations. 50 µl of extracts at different concentrations were taken in different test tubes. The volume was adjusted to 4 ml by adding MeOH and so the concentrations of extracts obtained from 1 µg/ml, 2 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml and 50 µg/ml. One milliliter of 0.1 mM methanolic solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at room temperature for 30 min in the dark. Absorbance was measured at 517 nm. Pure methanol was used for the baseline correction and distilled water (50 µl) as a control sample. Results were expressed as the inhibition percentage and were calculated using the following formula:

$$Inhibition = \frac{Ac - As}{Ac} \cdot 100\%$$

Ac = Control absorbance, *As* = Sample absorbance.

IC_{50} values (concentration of sample requires to inhibit 50 % of the initial amount of DPPH radicals) were calculated by using Origin Pro 7.

Reducing power assay (FRAP)

FRAP method is based on the ability of water soluble phenolic substances to reduce Fe^{3+} to Fe^{2+}. The resulting Fe^{2+} with TPTZ reagent (2,4,6-tri(2-pyridyl)-s-triazine) creates complex with maximum absorption at 593 nm. The reaction occurs in acidic conditions (pH 3,6). The method was performed according to standard procedure described by Benzie and Strain (1996) with slight modifications. Dry extracts were diluted with water to a concentration of 1 mg/ml. 150 µl of dissolved sample was added in 4,5 ml FRAP solution consisting of 25 ml of acetate buffer (pH 3,6), 2,5 ml of TPTZ solution (10 mmol) in HCl solution (40 mmol) and 2,5 ml of $FeCl_3*6H_2O$ solution (20 mmol). Absorbance was measured after 5 min at 593 nm and results were expressed as mmol Fe^{2+} per liter of the extract solution. Freshly prepared solution of $FeSO_4*7H_2O$ of different concentrations (200, 400, 600, 800 i 1000 µmol/l) was used for the standard curve constructing.

Statistical analysis

Statistical analyses were performed in Origin pro 8. All the analyses were carried out in triplicate, and the results are shown as mean value and standard deviation. The data were analyzed by analysis of variance (one-way ANOVA), and were compared by Tukey's difference test; the 1% level being used to determine the significance.

RESULTS AND DISCUSSION

In the recent years there is an increased interest in obtaining polyphenolic antioxidants from spent coffee residues (Mussatto et al., 2011; Murthy and Naidu, 2012). However, the number of available information on the application of fermentation to enhance biological activity of spent coffee extracts is still limited.

Orozco et al. (2008) examined the influence of fermentation by different strains of *Streptomyces* sp. on the polyphenol content in the waste coffee pulp. They concluded that some strains of *Streptomyces* reduce polyphenol content up to 30,5 %, which improves the properties of waste pulp for use as animal feed.

The coffee pulp fermentation by strains of fungi *Aspergillus tamarii* was reported by Arellano-Gonzalez et al. (2011). A solid-state fermentation was applied and extraction was performed by aqueous methanol. It was found that the total polyphenol content does not differ significantly between fermented and nonfermented samples. However, fermentation increased the concentration of free polyphenols in relation to the covalently linked polyphenols, especially free hydroxicinamic acid which led to increased antioxidant activity of fermented samples. Machado et al. (2012) investigated the possibility of coffee waste solid-state fermentation by different strains of fungi (*Aspergillus*, *Mucor*, *Penicillium*, and *Neurospora*) and their influence on the concentration of released polyphenols. It was found

that this type of fermentation could be promising method for obtaining polyphenols without using of chemical solvents.
In our work, spent coffee extracts fermented by different strains of *Streptomyces* sp. was used to determine their effect on yield, content of polyphenols and antioxidant activity of obtained extracts. Extraction yield is expressed as a percentage (g dry matter of obtained extract per 100 g dry matter of spent espresso coffee) and total polyphenol content is expressed as mg GA per g dry matter of the extract. DPPH radical scavenging activity was expressed by IC_{50} values (μg / ml), while the FRAP values are expressed in mmol / l of extract solution (concentration 1 mg / ml). The results of these determinations are summarized in Table 1.

Table 1 Total polyphenol content and IC_{50} values in coffee extracts samples

Sample	Yield (%)	Total polyphenol content (mg GAE*/g d.m.)	IC_{50} (μg/ml)	FRAP values (mmol Fe^{2+}/l) **
Control	3.657	89,35±0,19 [a]	24.81±0.22 [a]	1.31±0.03
CKS1	5.150	86,01±0,14 [b]	32.82±0.17 [b]	1.22±0.03
CKS5	4.187	94,27±0,11 [c]	24.57±0.08 [a]	1.42±0.11
CKS7	5.006	89,72±0,14 [a]	27.73±0.12 [c]	1.41±0.21
CKS8	4.208	88,67±0,15 [d]	25.09±0.13 [a]	1.25±0.08
CKS18	4.780	99,93±0,14 [e]	23.54±0.09 [d]	1.50±0.11

*GAE – gallic acid equivalents
** Sample concentration is 1 mg/ml
Results are expressed as the mean value±standard deviation on dry matter basis;
[a, b, c] the same letter indicates no significant difference ($p<0.01$)

According to the obtained results, fermentation increases the total yield of extraction in all samples. However, total polyphenol content and antioxidant activity are strain depended. Fermentation by *Streptomyces* strains CKS 5, CKS 7 and CKS 18 increase the polyphenol content, compared to the nonfermented control sample. On the other hand, coffee samples fermented by strains CKS 1 and CKS 8 showed a decrease in polyphenol content, compared to the nonfermented control sample. It should be noted that the higher content of polyphenols was detected in samples obtained by strains which produce dark brown pigment (probably melanin) in growth medium. It is known that some strains of *Streptomyces* produce melanins, which show different biological properties including antioxidant (Diraviyam et al., 2011). Thus, the presence of melanin could be the reason of increasing of polyphenols in extracts of fermented spent coffee.

CONCLUSIONS

Spent coffee produced during espresso coffee preparation contain significant amounts of biologically valuable ingredients which may be a good source of natural antioxidants. Our results indicate that fermentation of spent coffee by *Streptomyces* strains could be useful for modulating its biological properties. Some strains increase the content of total polyphenols in the extracts and their antioxidant properties respectively. These strains could be applied to obtain dietary supplements with antioxidant activity. On the other hand, some strains affect the reduction of total polyphenol content in spent coffee extracts, which can be used for their utilization as feed components. In any case, the spent coffee fermentation by selected strains of *Streptomyces* can help to improve the properties of final products and its potential application in the food and pharmaceutical industries.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31035 “Application of biotechnological methods for sustainable exploitation of by-products of agro-industry” financed by the Ministry of Science and Technological Development, Republic of Serbia.

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ENCAPSULATION AND RELEASE PROFILES OF CAFFEINE FROM MICROPARTICLES

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ABSTRACT: The purpose of this study was to encapsulate caffeine in alginate-chitosan cross-linked microparticles enriched with ascorbic acid by applying electrostatic extrusion technique. Three different forms of caffeine were encapsulated: liquid extract of caffeine from the plant guarana (*Paullinia cupana*), food-grade solid caffeine and analytical-grade caffeine. High performance liquid chromatography (HPLC-PDA) was used to evaluate the release kinetics of caffeine from microbeads in water, while the corresponding antioxidant capacity was evaluated by applying the ABTS radical scavenging assay. Scanning electron microscopy and laser diffraction particle size determination were used to provide information about the physical properties of microparticles. The microbeads encapsulating caffeine were uniformly sized spheres of about 600-800 μm. The encapsulation efficiency of all microparticles ranged between 70-80%. Caffeine was mainly released within 10-15 min, depending on the used caffeine form, while the ascorbic acid was relatively rapidly released from microbeads according to antioxidant capacity exhibited in water. The obtained results suggest that electrostatic extrusion can be applied for the entrapment of caffeine in alginate-chitosan microbeads, while the addition of ascorbic acid further enhances the antioxidant activity of such obtained microcapsules.
Key words: *alginate, antioxidant capacity, ascorbic acid, caffeine, chitosan, encapsulation*

INTRODUCTION

In the past decade, increased progress has been achieved towards the production of microencapsulated therapeutic products and formulations, and their commercialization. Micro- and nanoparticles have the potential to revolutionize food production and nutrition, and modify modern drugs and phytochemicals by increasing their efficacy, stability, and solubility, decreasing their toxicity and sustaining their release, thus enabling advanced pharmacological effects.

Food-based materials that are usually used as carriers for controlled drug-release include whey protein, gum arabic, gelatin, alginate, pectin and chitosan (Deyao et al., 1993; Wang et al., 2004; Gunasekaran et al., 2007; Joseph and Venkataram, 1995; Ramakrishnan et al., 2007). Alginate is a water-soluble linear polysaccharide, which forms gels in aqueous solutions in the presence of divalent ions, such as $Ca^{2+,}$ due to the creation of intermolecular cross-links with the carboxyl groups. However, the effectiveness of Ca^{2+} alginate microspheres as drug carriers is mainly limited by the hydrogel high porosity, responsible for possible burst effect or too fast release of the entrapped drug, as well as for low drug loading ability due to drug leakage through the pores during their preparation (Matricardi et al., 2008). Combination of Ca^{2+} alginate with chitosan, by interaction between the free carboxyl groups of the first and the amino groups of the second one, has proven to be a successful strategy not only to overcome these drawbacks, increasing the gel mechanical properties and reducing its permeability, but also to combine the favourable properties of both polymers, improving their ability as carriers for achieving a specific drug delivery (Matricardi et al., 2008; Wittaya-Areekul et al., 2006; Mladenovska et al., 2007).

Hydrogel beads are usually produced by a so called dropping method which consists of droplet formation by pumping of a solution through a needle and further solidification of the falling droplets in a hardening solution via counter ion exchange or thermic gelling. Most studies have been conducted with large alginate beads (2-3 mm) produced by droplet formation from a syringe under gravity or under coaxial air flow (Klein et al., 1983). The application of electrostatic extrusion for production of microparticles and entrapment of biologically active compounds has been investigated more extensively in recent years. This encapsulation technique introduces high electrostatic potentials between the needle and the hardening solution and thus greatly improves droplet formation and production of small beads (0.1-1 mm). Moreover, among different encapsulation techniques, electrostatic extrusion provides uniformly sized gel microbeads (Bugarski et al., 1993; Bugarski et al., 1994a; Bugarski et al., 1994b), with the advantage of forming small particles (≥ 50 μm)
In recent years, caffeine has received increasing attention in food and pharmaceutical industries, due to its pharmacological properties which comprise stimulation of the central nervous system, peripheral vasoconstriction, relaxation of the smooth muscle and myocardial stimulation (McLellan et al., 2005). The intake of caffeine by consumption of coffee, tea, cola and energy drinks is widely spread by consumers with the aim of overcoming sleep deprivation and enhancing sport performance. As caffeine absorption from the gastrointestinal tract is rapid and reaches 99% in humans about 45 min after ingestion (Blanchard and Sawers, 1983), efforts to control the release of caffeine after ingestion may be beneficial for food and nutraceutical applications. So far, attempts to entrap caffeine by microencapsulation have been conducted using combined cross-linking treatments of bovine serum albumin gel beadlets (Gan et al., 2009), hydrogenated and fluorinated gel emulsions and cubic phases (Fa et al., 2004) and concentrated W/O emulsions (Clément et al., 2000), while alginate-chitosan hydrogel has not been used yet as a carrier for encapsulation of caffeine performed. Therefore, in this study the electrostatic extrusion technique was applied to immobilize caffeine in alginate-chitosan cross-linked gel microbeads and, duration of release and the quantity of the drug released was examined.

MATERIALS AND METHODS

Preparation and characterization of alginate-chitosan microcapsules

Three different forms of caffeine were evaluated for immobilization in alginate-chitosan microbeads; liquid extract of caffeine obtained from the plant guarana (*Paullinia cupana*), solid caffeine isolated from a natural source for food purposes (Döhler, Darmstadt, Germany) and analytical standard of caffeine for drug analysis (Sigma Aldrich, USA). Alginate solution (1,5% w/v) was prepared by dissolving a low viscosity sodium alginate Protanal LF 20/40 (purchased from FMC Biopolymer) in previously prepared caffeine solutions in water (2% (v/v) of liquid guarana extract, 2% (w/v) of solid caffeine for food purposes and 3% (w/v) of analytical standard of caffeine. Each mixture was well homogenized and drawn into a 5 ml syringe attached to 23-gauge metal needle with a blunt tip. The syringe was placed in a syringe pump (Razel, Scientific Instruments, Stamford, CT) and a high voltage dc unit (Model 30R, Bertan Associates, Inc., New York) was applied to control the potential difference. The solution was extruded at a voltage of 6.3 kV and flow rate of 25.2 cm^3/h to a receiving beaker containing cross-linking solution which was constituted of 50 ml 2% (w/v) calcium chloride solution, 0.5% (w/v) chitosan (molecular weight: 100 000-300 000) and 2% (w/v) of ascorbic acid dissolved in the previously prepared caffeine solutions (the final pH of receiving solutions was 2.65). The microparticles were left in the solution for 24h and then washed several times with the respective caffeine solution.
Shape and surface morphology of microspheres were examined using a FEI Quanta200™ environmental scanning electron microscope (ESEM), with a spatial resolution at 30 kV of 3 nm (FEI Company, Oregon, USA). Particle size of the obtained microparticles was determined using Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) equipped with the Hydro 2000S dispersion unit.

Encapsulation efficiency
The amount of caffeine entrapped in the microparticles was estimated by dissolving a known amount of capsules in sodium citrate (2% w/v) during 60 min by vigorous shaking on a Vortex mixer (Tehnica, Železniki, Slovenia) at room temperature. The content of caffeine loaded in the beads was determined by a HPLC method described by Belščak-Cvitanović et al. (2011). The percentage of encapsulation efficiency was calculated according to the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{A}{A_0} \times 100$$

where A is the caffeine content determined after destabilization of the microparticles with the sodium citrate solution and A_0 is the caffeine content of the initial extract.

Release kinetics in water
The release of caffeine from the alginate-chitosan microbeads in water was followed in terms of quantifying the caffeine content by HPLC analysis and determination of antioxidant capacity by using the ABTS assay (described in the following section). For the analysis a known amount of particles (~ 3g) was suspended in 50 mL of distilled water. The samples were submitted to continuous agitation on an orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ) operating at 100 rpm. At certain time intervals, an aliquot of the supernatant was taken for analysis and replaced by the same amount of fresh distilled water. The experiments were performed in triplicate.

Determination of antioxidant capacity
The Trolox equivalent antioxidant capacity (TEAC) was estimated by the ABTS radical cation decolorization assay (Re et al., 1999). The results, obtained from triplicate analyses, were expressed as Trolox equivalents, and derived from a calibration curve determined for this standard (100-1000 µM).

RESULTS AND DISCUSSION

Table 1 shows the encapsulation efficiency of caffeine in alginate-chitosan microbeads. The highest encapsulation efficiency was obtained by using liquid extract of caffeine derived from guarana (81.50%), while the lowest was observed for 3% solution of standard of caffeine (71.61%).

Table 1. Encapsulation efficiencies and particle size of alginate-chitosan microbeads encapsulating three different caffeine forms.

	2% guarana caffeine	**2% food caffeine**	**3% standard caffeine**
Encapsulation efficiency (%)	81.50 ± 2.48	76.45 ± 3.25	71.61 ± 4.52
Particle size range (µm)	620 – 750	670 - 750	700-850

Results are given as mean ± standard deviation (n = 3)

As it can be seen, the encapsulation efficiency is on average 79% when 2% caffeine is encapsulated in microbeads, and ~76% in case of caffeine standard which is added in 3%. Particle size of the microbeads was inversely related to the encapsulation efficiency: the microbeads encapsulating 2% of guarana caffeine exhibited the lowest particle size, while the microbeads encapsulating 3% of standard of caffeine were the largest. Namely, caffeine exhibits moderate solubility in water (about 2g/100ml at room temperature), and when 3% solution of caffeine was used for the preparation of microbeads, saturation was achieved, followed by crystallization of caffeine. The outcome of this process was the presence of caffeine crystals which may have resulted with larger particles and lower encapsulation

performance. This can also be observed on SEM micrographs of the produced microbeads shown in figure 1. The microbeads encapsulating 2% guarana caffeine were round, while the microbeads obtained from the dissolved caffeine were irregular in shape (2% of food caffeine) or aggregated (3% of standard of caffeine).

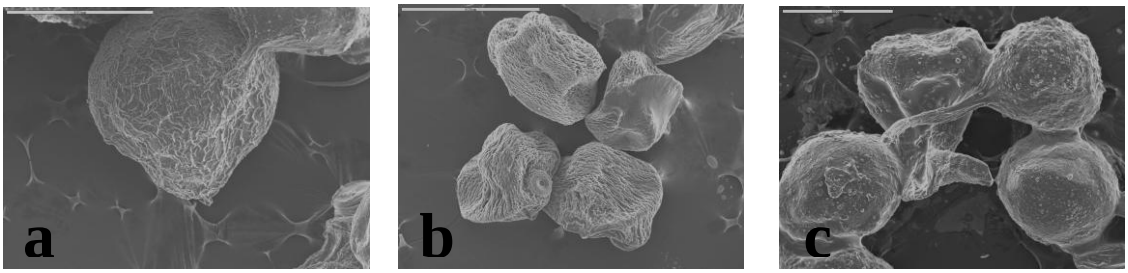

Figure 1. SEM micrographs of alginate-chitosan microbeads encapsulating a) 2% guarana caffeine, b) 2% food purposes caffeine and c) 3% of caffeine standard.

Release of the entrapped caffeine from alginate-chitosan microbeads also depended on the caffeine form and concentration. As it can be seen, the major content of caffeine contained in the microbeads was released within the first 10-15 minutes from microbeads encapsulating 2% of guarana caffeine and 3% of caffeine standard, followed by prolonged release of the rest, up to 24h (figure 2). Surprisingly, the microbeads encapsulating 2% of caffeine for food purposes exhibited a very rapid release of caffeine in water and after 2 min, the concentration of caffeine reached a steady state.
However, despite the rapid release of caffeine, it was observed that the amount of caffeine released from the microbeads was notably lower compared to the other two encapsulated caffeine forms. Poor diffusion of food-grade caffeine indicates the potential of using alginate-chitosan for the encapsulation of caffeine for food purposes, since it enables better retention of caffeine.

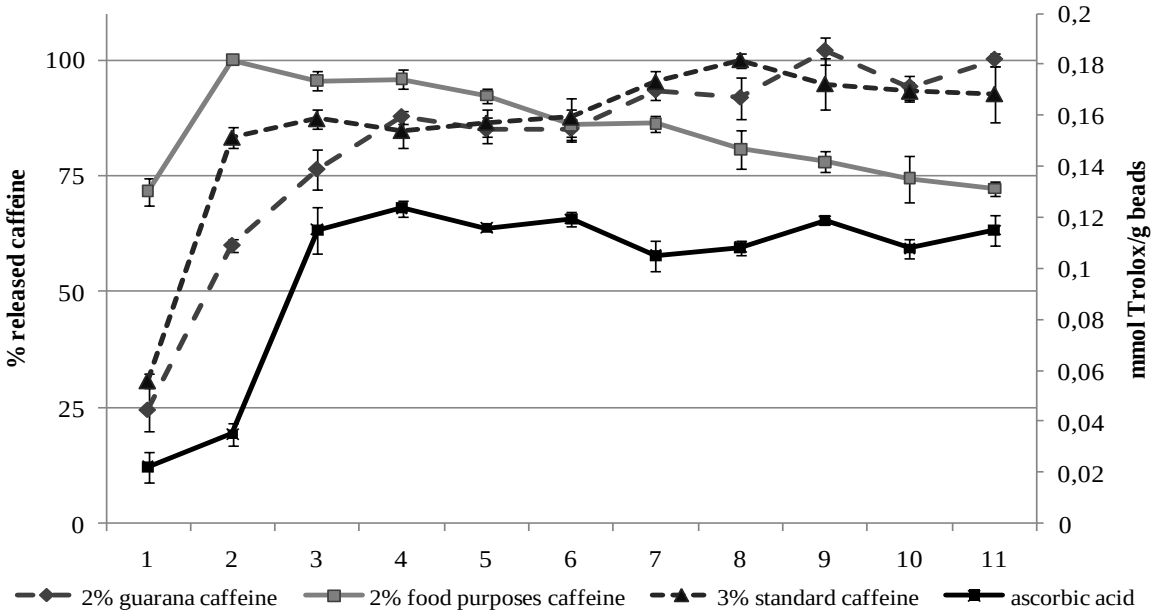

Figure 2. Release profile of caffeine and antioxidant capacity of ascorbic acid from the alginate-chitosan microparticles.

Kinetics of ascorbic acid release was followed in terms of antioxidant capacity detected in water as a function of time (figure 2). Ascorbic acid was used in order to ensure low pH necessary for the dissolution of chitosan, which additionally enhanced the bioactivity and antioxidant capacity of obtained microparticles. Alginate-chitosan cross-linked microbeads

enhanced with ascorbic acid are already approved as suitable for encapsulation of polyphenols derived from different medicinal plants (Belščak-Cvitanović et al., 2011). According to the results, the microbeads obtained in this study exhibited significant antioxidant capacity, and release of caffeine occurred simultaneously with the release of ascorbic acid which ended within 5 min.

CONCLUSIONS

Alginate-chitosan crosslinked microparticles obtained by electrostatic extrusion can be used for the encapsulation of caffeine regardless of the caffeine origin and concentration. High encapsulation efficiency of caffeine was obtained for all caffeine forms, while caffeine for food purposes was retained in the highest amount in the particles when subjected to release in water. The evaluated combination of encapsulating agents provides a potentially effective way to entrap caffeine and exhibit antioxidant capacity due to applied ascorbic acid.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science, Education and Sports, Republic of Croatia (Project No. 058-0000000-3470 and Croatia-Serbia Bilateral agreement 2009-2010), as well as by the Ministry of Science and Technological Development, Republic of Serbia (National Project No. III46010 2011-2014 and Serbia-Croatia Bilateral agreement 2009-2010).

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ENCAPSULATION OF NATURAL ANTIOXIDANT RESVERATROL IN LIPOSOMES

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ABSTRACT: Liposomes have been shown to be suitable systems for encapsulation and preserving the health–beneficial properties of a wide range of biological active ingredients such as resveratrol (RSV). The aim of this study was to encapsulate RSV in liposomes, with a goal to achieve the extended release and improved stability of RSV. Multilameral liposomes were prepared by means of two different methods: thin film method (TF) and proliposome method (PRO). In both methods, the ratio between added RSV and phospolipon 90G (P90G) was 1:20 w/w. Extrusion and sonication were applied in order to obtain unilameral liposomes. Both methods were efficient in capturing RSV within the microparticles, thus encapsulation efficiency had high values (92,9% in case of TF and 97,4% in case of PRO). The size reduction of liposomes resulted with particles of the average diameter ranged between 120 and 270 nm. Antioxidative activity was retained at a high level (approximately 95%). Franz diffusion cell was used for release studies and diffusion of RSV was monitored for 6h. According to the results, liposomes appeared to be suitable vehicles for encapsulation of resveratrol where PRO is particularly useful for encapsulation of antioxidants.
Key words: *encapsulation, liposome, resveratrol, release study*

INTRODUCTION

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods. They can scavenge free radicals and slow down the process of lipid peroxidation. Antioxidants can also protect human body from free radicals and block the progress of many different chronic diseases (Aruoma et al., 1998). RSV has been shown to possess a remarkably strong antioxidant activity, even stronger than vitamins E and C in some assay systems (Stojanović et al., 2001). It exists in two structural isomeric forms, *cis* and *trans*, but the *trans*-resveratrol is more active than the *cis* isomer (King et al., 2006).

At least 72 plant species contain RSV and it is known that plants produce RSV in response to different kinds of stress factors (Jang et al., 1997). The primary dietary sources of RSV in human diet are peanuts, berries, grapes and wine but it is also reported that coco and chocolate contain RSV too (Counet et al., 2006). It is considered that increased consumption of food which contains RSV may improve health. Some authors have presented that RSV displays wide pharmacological activities such as antioxidant, anti-inflammatory, analgesic, cardioprotective, neuroprotective, chemo-preventive and antiaging activities (Baur and Sinclair, 2006).

Though, this positive effect of RSV is very restricted because RSV is easily oxidizable and photosensitive compound (Pineiro et al., 2006). Furthermore, RSV is highly lipophilic, poor soluble in water and has rapid metabolism, thus it is very hard to use RSV in native form as a food additive or food supplement (Walle et al., 2004; Lopez-Nicolas et al., 2006). All this drawbacks of RSV can be overcome to some extent by microencapsulation.

Liposomes approved to be suitable vehicles for encapsulation and delivery of wide variety of substances (Peschka et al., 1998; Gibbis et al., 1999). Liposomes are spherical particles, consisting of a membrane. The membrane is made up of phospholipid bilayer which is

similar to the lipid fraction of cell membranes. If liposomes have just one bilayer they are called small (<30 nm) or large (30–100 nm) unilamellar vesicles (SUV and LUV). Those which contain more than single bilayer membrane are called multilamellar vesicles (MLV)
The aim of this study was to encapsulate RSV in liposomes, with a goal to achieve the extended release and improved bioavailability of RSV. Furthermore, *in vitro* releasing properties of RSV was investigated to show potential of loaded liposomes to be used as an additive to functional food.

MATERILAS AND METHODS

Preparation of liposomes

P90G (phosphatidylcholine 92–98% pure, supplied by Natterman Phospholipids, Germany,) was used for liposome preparation. It provides liposomes which are highly bioavailable (Pjanović et al., 2010). Both, empty liposomes and liposomes loaded with RSV were prepared by two different methods.
The first method was thin film method (TF) and liposomes were prepared as follows: 1,4g of P90G, 0,6g of cholesterol (Cholesterol, Sigma–Aldrich, Sigma Grade, ≥99%) and 0,07g of RSV (Resveratrol, ChromaDex, California) were dissolved in ethanol in a round-bottomed flask (Lasch et al., 2003). The solvent was completely removed on a rotary vacuum evaporator (BUCHI Rotavapor R-114), at 45^{o}C and pressure of 175 mbar. The resulting film was further dried for 15 min and then hydrated by adding an aqueous phase (100 ml of distilled water). The dispersion was hand-shaken for 20 minutes. In the same way was prepared liposomal dispersion without RSV, used for computation.
The second method of liposomes preparation, PRO method, was derived according to literature data (Perrett et al., 1991) with slight modifications. A mixture of 2g P90G, 0,1g of RSV and ethanol was stirred and after adding a small quantity of distilled water, heated to 60 ^{O}C for a few minutes. After cooling to room temperature, aqueous phase (100 ml distilled water) was added in small parts. The suspension was stirred for one more hour at 800 rpm. In this case liposome dispersion without RSV was also prepared using the same procedure
All samples prepared as described above were MLV dispersions. In order to get LUV and liposomes with smaller diameter, two different techniques were applied: extrusion and sonication.
The extrusion technique is based on forced passes of dispersed phase containing MLV through a membrane with a uniform pore size distribution. These passages generate a homogeneous population of smaller vesicles (Gregoriadis, 2006). In this study 1ml of each sample was extruded 11 times through polycarbonate membranes with decreasing pore sizes (800 nm, 400 nm, 200 nm and at the end 100 nm) using the LiposoFast Basic extrusion device (Avestin, Ottawa, Canada).
Application of high-intensity ultrasound to liposome dispersions can result in formation of unilamellar vesicles (Gregoriadis, 2006). For this purpose a probe was directly immersed in 10 ml of each sample. Sonication was achieved with Ultrasonic Processor VCX 500 W (Cole Parmer, Vernon Hills, IL); amplitude was 40 % and duration 360s. During whole process samples were constantly cooled with ice (Lasch et al., 2003).

Size measurements and stability of liposome dispersions

The size of the liposomes (average diameter), polydispersity indexes (PDI) and zeta potential were determined by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Each sample was measured three times at room temperature and the average values were taken as results. The measurements were repeated on the 1st, 3rd, 7th, 14th and 21st day after preparation of dispersions in order to examine stability of the liposomes. The dispersions of liposomes were storage in the refrigerator at about 4°C.

Encapsulation efficiency
In order to determined encapsulation efficiency (EE%) samples were centrifuged at 45000 rpm for 45 minutes at 10°C in a Thermo Scientific Sorval WX Ultra series ultracentrifuge (Thermo Scientific, Waltham, MA). The amount of RSV in supernatant was defined spectrophotometrically at 306 nm. EE% was calculated as the amount of RSV encapsulated in liposomes divided by the amount of RSV used for their preparation as shown in Equation 1:

$$EE\% = [(m_i - m_{sup})/m_i] \times 100 \quad (1)$$

where m_i is amount of RSV used for the preparation of liposomes and m_{sup} is amount of RSV determined in supernatant.

Determination of free radical-scavenging activity (ABTS)
This method was used for measuring the relative ability of RSV to scavenge the free 2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS•). This radical was formed by mixing 5 mL of ABTS water stock solution and 88 µL of potassium persulfate. Before use, the mixture was incubated at room temperature in the dark for 12 to 16 h. On the day of analysis, the ABTS• solution was diluted with ethanol to achieve absorbance of 0.70 (±0.02) at 734 nm. A 4 µL aliquot of a sample was added to 4,5 mL of the prepared ABTS• solution and the absorbance was measured after 4 min. The inhibition of ABTS• was estimated based on the method of Re et al., 1999 with a slight modification and calculated by the following equation:

$$\% \text{ inhibition} = [A_0 - (A_1 - A_s)/A_0] \times 100 \quad (2)$$

where A_0 is the absorbance of the control solution (containing only ABTS), A_1 the absorbance of the sample and A_s is the decrease of the absorbance of ABTS• caused by blank sample.

Release study
Jacketed Franz diffusion cell (donation of PermeGear, Inc., USA) was used for release experiments. The cell contains the donor and the receptor section which were separated with an acetate-cellulose membrane (pore size 0,45 µm). Receptor section has a fixed volume (20 ml) and contains a magnetic stirrer. Before the start of the experiment, the cell was thermostated with receptor fluid (ethanol 50%) for 30 minutes. As a donor phase was used 1ml of liposome dispersions with RSV which were prepared as described above. During the experiment 0,5 mL of a sample was taken from the receptor section and the same volume of fresh fluid was added back. Each experiment lasted 6 hours and concentration of RSV was determined spectrophotometrically, measuring the absorbance at 306 nm.

RESULTS AND DISCUSSION

For easier discussion in the table 1 is given an overview of the abbreviations which are going to be used further in the text for sample names.

Table 1. Overview of the abbreviations

Method for liposome preparation	Technique applied for size reduction (LUV)	Empty samples	Samples with RSV
Thin film	/	TF-MLV	RSV-TF-MLV
	Extrusion	TF-100	RSV-TF-100
	Sonication	TF-SON	RSV-TF-SON
Proliposome	/	PRO-MLV	RSV-PRO-MLV
	Extrusion	PRO-100	RSV-PRO-100
	Sonication	PRO-SON	RSV-PRO-SON

Stability of the liposomes was monitored during 21 days and the size of the liposomes, polydispersity index and zeta potential were measured on the 1st, 3rd, 7th, 14th and 21st day after preparation. The results showed that liposomes were uniform and an average diameter depended on the technique used for liposome size reduction. PRO-MLV were almost five times smaller than TF-MLV (Figure 1). Liposomes which were reduced by sonication had average diameter between 120 and 290 nm (results are not shown). TF-100, RSV-TF-100, PRO-100 and RSV-PRO-100 had average diameter between 150 and 270 nm, but the one with RSV have shown slightly smaller average diameters then empty liposomes (Fig. 2). If one compares liposomes with and without RSV, obtained by the same method, it is apparent that RSV reduced the size of liposomes. PDI values of all samples were smaller than 0,7 which means that liposomes had fine size distribution, therefore a good quality (Caddeo et al., 2008). We notice that the extrusion technique enabled to get more homogeneous distribution of particles which was also confirmed by Budai et al., 2004. The zeta potential was negative for all samples and RSV did not have any influence on this parameter. The results show that liposomes were physically stable during 21 days of storage (significance was tested at the 0,05 levels of probability).

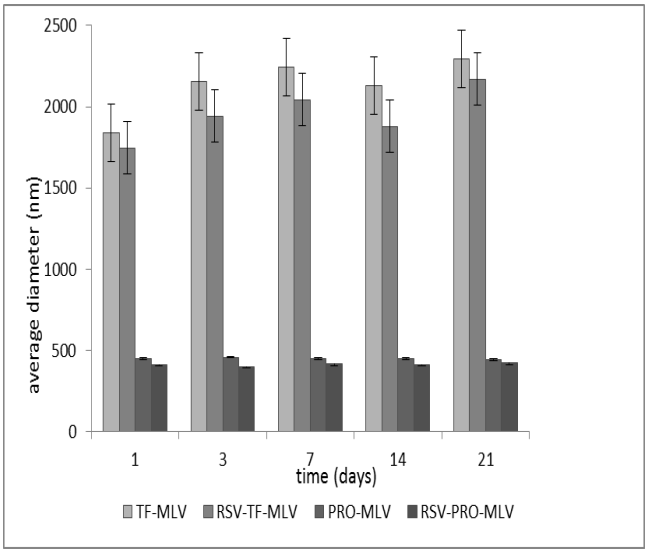

Figure 1. Average diameters of multilamelar liposomes during 21 days of storage, (error bars denote S.D., *n*=3)

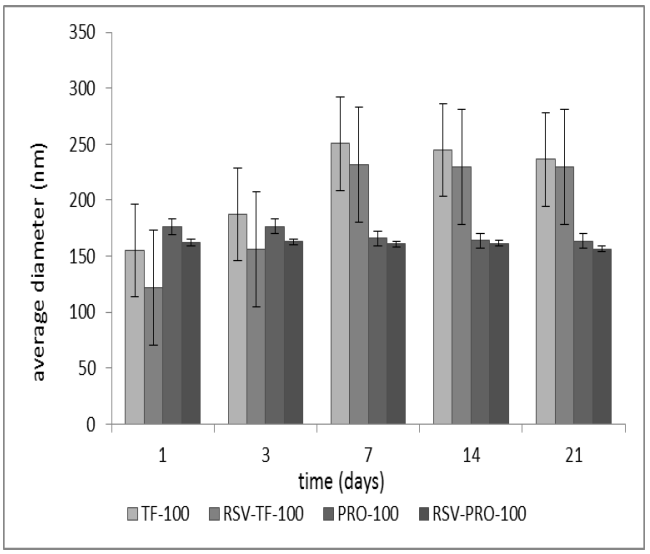

Figure 2. Average diameters of LUV liposomes obtained by extrusion technique during 21 days (error bars denote S.D., *n*=3)

The results of encapsulation efficiency of RSV in liposomes are shown in Table 2. As it can be seen, PRO method provided better EE% for MLV then TF method. The same result was noticeable for RSV-PRO-SON and RSV-PRO-100. The results suggest that during sonication there was significant loss of RSV, probably because of high shear forces on MLV, which caused disruption of the MLV structures and leakage of RSV from liposomes. The encapsulation efficiency of RSV in liposomes treated by extrusion through membranes was the same as for MLV (prior to extrusion). The results of encapsulation efficiency show great advantage of extrusion technique over the sonication

Table 2. Encapsulation efficiency (EE %) of RSV in liposomes and relative antioxidative capacity (RAC%) of RSV loaded liposomes to scavenge ABTS• free radical

Sample	Thin film method		Proliposome method	
	EE%	RAC%	EE%	RAC%
RSV-MLV	92,88±0,27	88,38±5,22	97,36±2,00	90,35±1,74
RSV-SON	44,22±1,26	38,86±1,46	55,88±2,81	40,07±4,27
RSV-100	92,27±0,82	96,55±1,17	96,08±0,62	94,66±0,57

Results are given as mean ± standard deviation (n = 3)

The relative antioxidative capacity of RSV is also given in Table 2. Dilution of RSV in ethanol was prepared in the same concentration as it was in liposomes samples and this solution (control sample) was also tested by the ABTS assay. Compared to control sample antioxidant activity of RSV was preserved at a high level. Only in case of LUV obtained by sonication technique relative antioxidative capacity of RSV was low. The reason for this result could be local overheating during sonication and loss of antioxidant activity of RSV at high temperatures.

The diffusion of RSV from liposomes was monitored during 6 hours using Franz diffusion cell. Solution of RSV in ethanol in appropriate concentration (control solution) was used for comparison. Four samples were chosen to be monitored (RSV-TF-MLV, RSV-PRO-MLV, RSV TF-100 and RSV-PRO-100) as they exhibited high EE% and consistent antioxidant activity. According to our results (Fig. 3) diffusion of RSV (from control solution) occurred rapidly and concentration of RSV in acceptor compartment reached maximum approximately after 100 minutes. RSV diffusion from liposomes was slower than diffusion of free RSV and RSV reached final concentration in acceptor compartment after more than 200 minutes. The results show that liposomes could be used for prolonged release of RSV which is important concern in real food applications. MLV liposomes were able to better retain RSV than LUV: in comparison after 100 min, ~50% of RSV was released from PRO-MLV and ~80 % from TF-100. This is directly related with size of the liposomes. Namely, MLV are of the one order larger than LUV (Fig. 1 and 2) and thus provide longer path for diffusion.

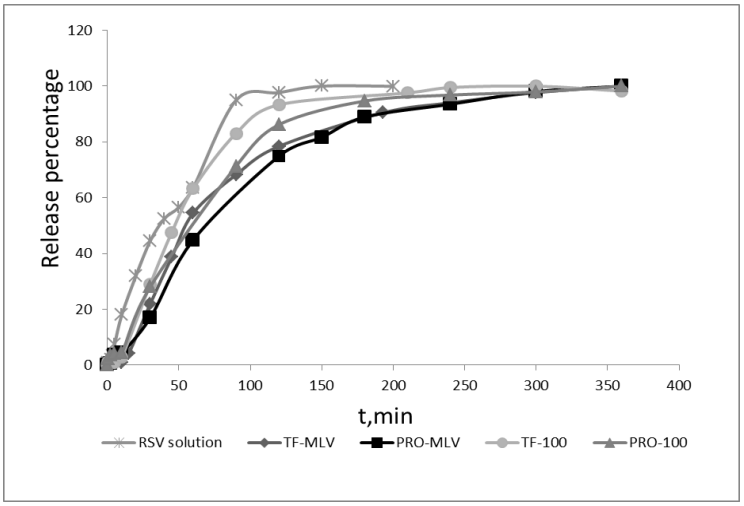

Figure 3. The diffusion of RSV from the solution and liposome dispersions

CONCLUSIONS

Two methods were used for preparation of RSV loaded liposomes: proliposome and thin film method. High encapsulation efficiency was achieved using both methods and the obtained liposomes were physically stabile during 21 days. According to the results, extrusion technique was shown as more suitable then sonication technique for size reduction of MLV. It also enabled more homogeneous distribution of liposomes. Antioxidative activity of RSV loaded in liposomes was retained at a high level. The diffusion experiments of RSV from liposomes indicated that liposomes could be used for prolonged release of RSV. Formulations based on proliposomes were easy for preparation and have shown good results, thus they are suggested as useful for encapsulation and controlled delivery of functional food ingredients.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education and Science, Republic of Serbia (Project No. III46010 and Serbia–Slovenia bilateral agreement 2010-2011).

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ENCAPSULATION OF *THYMUS SERPYLLUM* L. AQUEOUS EXTRACT IN CHITOSAN AND ALGINATE - CHITOSAN MICROBEADS

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ABSTRACT: The aim of this study was to investigate encapsulation processes of *Thymus serpyllum L.* aqueous extract in chitosan and alginate-chitosan microbeads and its consecutive release. Chitosan microbeads were prepared in water-in-oil (W/O) microemulsion, while alginate-chitosan microbeads were prepared by electrostatic extrusion. The outer surface of microbeads was observed by optical microscopy. The interaction between chitosan or alginate-chitosan microbeads and extract compounds were analyzed by FT-IR. Release profiles of polyphenolic compounds from the microbeads in water was monitored. Experimental results show that the applied methods gave chitosan and alginate-chitosan microbeads with an average diameter of 390 μm and 890 μm, respectively. FT-IR analyses confirmed the presence of polyphenolic extract within the synthetized microbeads. The encapsulation efficiency was determined with respect to total polyphenolic content. Chitosan microbeads showed lower encapsulation efficiency of ~10%, but prolonged release, compared to alginate–chitosan microbeads where encapsulation efficiency was ~70%. The uniformity of the obtained microbeads was confirmed by optical microscopy. This study showed that, by some synthesis modification of both, chitosan and alginate-chitosan microbeads have the potential to be used for encapsulation of aqueous phenolic extracts.
Key words: *encapsulation, alginate, chitosan, polyphenols, release study*

INTRODUCTION

One of the current focuses of the life science community is naturally derived antioxidants, especially plant polyphenols, which exhibit high antioxidant properties (Stojanovic et al., 2011). One of the popular herbs rich in polyphenols is thyme (*Thymus serpyllum L.*). A wide array of positive health effects has been described to plant polyphenols, such as antimicrobial properties and the ability to protect against cancer and cardiovascular diseases. Plant polyphenols are usually consumed by preparing a tea or herbal infusion through an aqueous extraction process. However, in this way active compounds can be easily affected by detrimental effects of oxygen, moisture or incompatible compounds during storage or consumption (Fang&Bhandari, 2010). This problem can be solved by applying the microencapsulation technology in order to improve the stability of extracted compounds during transport, storage or processing. Moreover, the goal is to achieve superior handling of the active compounds by conversion of liquid active compounds into solid forms, which in addition can be used as food additives or food supplements.
Among numerous entrapping materials used for the encapsulation of a targeted substance, sodium alginate and chitosan have received much attention in drug delivery system for their excellent biocompatibility (Xu et al., 2007). Among polyanionic polymers, alginate has been widely studied and applied for its possibility to modulate the release, according to the properties of its carboxyl groups as well as its biodegradability and absence of toxicity. Chitosan also has wide applications in pharmaceutical technology as tablet disintegrant, for

the production of controlled release solid dosages (Illum, 1998; Kas, 1997; Sezer&Akbuga, 1999) or for improving of drug dissolution (Gupta&RaviKumar, 2000). Much attention has been given to the use of chitosan-alginate polyelectrolyte complex in controlled drug delivery (Lee&Park, 1996). The use of chitosan has been reported in the literature for coating alginate beads in order to control the diffusion rate of the encapsulated substances and also as an additive for the bulk modification of the bead structure (Anal&Stevens, 2005).
In this study, alginate-chitosan and chitosan microbeads were used for encapsulation of polyphenolic extract of *Thymus serpyllum L.* The release profile of encapsulated polyphenols from both types of microbeads was monitored and compared. The microbeads size, as well as the morphology and the interactions between chitosan or alginate-chitosan microbeads and extract compounds were analyzed.

MATERIALS AND METHODS

Preparation of thyme aqueous extract

Thyme (The Institute for Medicinal Plant Research "Dr Josif Pancic", Belgrade, Serbia) extract was prepared by conventional water extraction, by pouring 200 mL distilled water over 10 g of ground plant at room temperature. The extraction was carried out for 30 min with stirring, and after that the obtained extract was filtered through medical gauze.

Preparation of alginate-chitosan microbeads

Medium viscosity alginate (Sigma-Aldrich, St. Louis, MO, USA) was used to obtained alginate-chitosan microbeads by electrostatic extrusion technique according to Belscak-Cvitanovic et al. (2011).

Preparation of chitosan microbeads

Firstly, 2% (w/v) chitosan solution was prepared by dissolving chitosan (*Fluka*, Japan) powder in acetic acid (HAc). Then, *n*-hexane as continuous phase, *n*-hexanol as aided surfactant, and HAc solution containing chitosan were mixed in a flask at the volume ratio of 11:6:4. The microemulsion was formed by adding Tween 80 into the mixture, drop by drop while stirring, until the mixed emulsion became semi-transparent. Chitosan microbeads were prepared by emulsion cross-linking method, slightly modified, according to Denkbas&Odabasi (2000). Briefly, chitosan aqueous solution was emulsified by mechanical stirring (1000 rpm) in oil phases containing surfactant. After 30 min of stirring, the obtained microbeads were solidified by cross-linking with 5% (w/v) glutaraldehyde (related to the chitosan solution volume), and the mixture was stirred for 1 hour more. Then the microbeads were filtered and washed three times with ethanol and distilled water and, at the end, with 0.1 M potassium phosphate buffer of pH 8. Then, microbeads were left to dry under vacuum, after which they were kept in desiccators. Dried chitosan microbeads (about 0.5 g) were placed in a flask containing 30 mL of freshly prepared thyme extract containing 2% (w/v) ascorbic acid for about 24h. During that time polyphenols from thyme extract were absorbed by chitosan microbeads.

Determination of total phenol content (TPC) of thyme extract

Total phenol content was determined spectrophotometrically using the Folin-Ciocalteau reagent (Kemika, Zagreb, Croatia), according to the method of Lachman et al. (1998). All measurements were performed in duplicate.

Encapsulation efficiency

Encapsulation efficiency (EE%) was calculated as the amount of TPC encapsulated in beads ($m_{TPC,e}$) divided by the TPC of the polymer-extract solution used for the preparation of beads (m_{TPC}), as shown in Equation 1:

$$EE\% = \frac{m_{\mathrm{TPC,e}}}{m_{\mathrm{TPC}}} \quad (1)$$

Alginate-chitosan microbeads: Quantification of polyphenolic compounds ($m_{\mathrm{TPC,e}}$) in microbeads was performed after dissolving beads in a sodium citrate solution (2 g/mL) (HiMedia Laboratories, Mumbai, India). Briefly, the fresh alginate-chitosan beads were homogenized with sodium citrate (in a weight ratio of 1:5) for 15 min under vigorous mixing using a vortex mixer to chemically dissolve them. The polymer-extract solution and polyphenolic-citrate solution were analyzed for TPC using the Folin–Ciocalteu method according to the procedure described in the section above.

Chitosan microbeads: Quantification of polyphenolic compounds ($m_{\mathrm{TPC,e}}$) in beads was calculated as difference between polyphenolic content in thyme extract (containing 2% of ascorbic acid) used for microbeads preparation and polyphenolic content in thyme extract after 24 h of absorption of polyphenols by chitosan microbeads. TPC was determined using the same procedure described in the section above.

Release studies

Release studies of polyphenols from freshly prepared hydrogel beads were performed at room temperature. About three grams of beads were placed in a flask containing 50 mL of water. The samples were submitted to continuous agitation on an orbital shaker. Successive aliquots of the surrounding aqueous medium (2 mL) were drawn from a water solution at proper time intervals. The content of polyphenols in aliquots was determined as previously described.

Fourier transform infrared spectroscopy

Powdered xerogel/KBr pastilles were submitted to FT-IR analysis and the spectra were obtained using a Bomem MB 100 FT-IR Spectrophotometer. The proper ratio (sample: KBr = 1:50) was mixed and grounded and then compressed into a pellet under a pressure of 11 t, for about a minute, using Graseby Specac Model: 15.011. Spectra were obtained in the 4000-400 cm^{-1} wave number range, at 25 ºC and at 4 cm^{-1} spectral resolution.

Morphology of the alginate-chitosan and chitosan microbeads

The alginate-chitosan and chitosan microbeads were characterized by optical microscopy (Olympus UC 30, USA). The volume mean diameters of the prepared microbeads, measured in triplicate for each batch, were determined in water (Pham at al., 2002). Particle size is expressed as volume mean diameter (μm) ± standard deviation (SD).

RESULTS AND DISCUSION

Table 1 shows the effect of matrix on TPC encapsulated or absorbed by 1g of microbeads. Chitosan microbeads exhibited higher TPC compared to alginate-chitosan, due to high sorption capacity of chitosan hydrogel. In our study, encapsulation efficiency (also presented in Table 1) is mainly influenced by preparation procedure (*in situ* encapsulation by electrostatic extrusion *versus* absorption method). In case of chitosan microbeads it is possible to increase encapsulation efficiency by reducing the expenditure of aqueous extract solution which is used for encapsulation. Future studies should be oriented toward optimization of microbeads-to-extract ratio which is applied during encapsulation by absorption method.

Table 1. Total phenol content (TPC) and encapsulation efficiency (EE) for alginate-chitosan and chitosan microbeads encapsulating *Thymus serpyllum L.* aqueous extract.

Type of matrix	TPC [mgGAE/g_{beads}]	EE [%]
Alginate-chitosan microbeads	11.89	68.02
Chitosan microbeads	23.83	9.65

Release studies

The release profiles for alginate-chitosan microbeads and chitosan microbeads encapsulating thyme extract are shown on Fig. 1. Most of the polyphenolic compounds were released relatively rapidly from alginate-chitosan beads and the curve plateau was reached after about 15 min. On the other hand, chitosan microbeads although smaller, showed somewhat better retainment of polyphenolic compounds, up to 50 min. Prolonged release of compounds from chitosan microbeads can be explained by less porous structure of chitosan matrix compared to alginate-chitosan structure (George&Abraham, 2006). The release-retarding property of chitosan may also be attributed to chemical interactions between polyphenolic compounds and hydroxyl and amino groups of chitosan matrix, as confirmed by FT-IR analysis described below. In case of both types of microbeads, decrease in size of microbeads occurred after release of extract compounds (Table 2), i.e. shrinkage of ~23 and ~35 vol% for alginate-chitosan and chitosan microbeads, respectively.

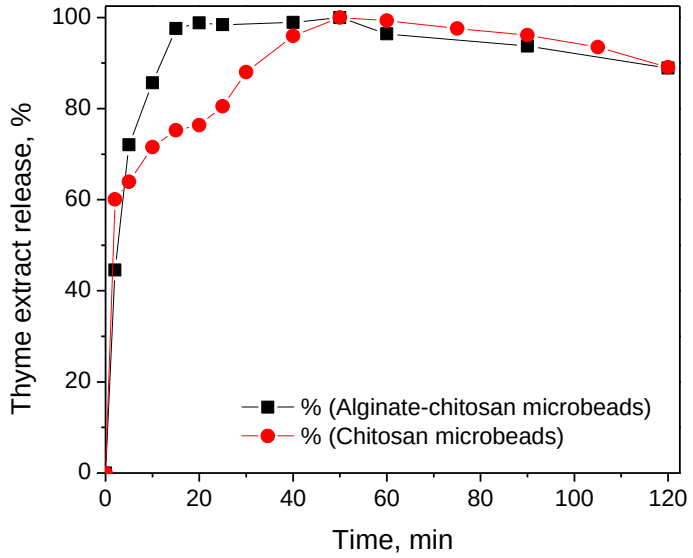


Figure 1. The release profiles for alginate-chitosan and chitosan microbeads encapsulating thyme extract.

FT-IR analysis

The FT-IR spectra of pure chitosan and alginate-chitosan microbeads, after encapsulation and release, were recorded and shown in Fig. 2. Those spectra are similar with several relevant peaks: between 3500 and 3000 cm^{-1}, corresponding to stretching vibration of free hydroxyl and N–H bonds in amino group, respectively, which are stronger in pure microbeads when compared to those with tea extract; the peaks at 2930.0 and 2880.1 cm^{-1} assigned to the asymmetric and symmetric mode of CH_2, respectively; the peaks at 1654 and 1595.4 cm^{-1} corresponding to the amide I and amide II band, respectively. As it is expected, extract encapsulation causes some changes in the peak position and intensity. The peak at 1092.0 cm^{-1} in the spectrum of pure alginate-chitosan is shifted to the lower wave length, 1073.0 cm^{-1}, while three peaks, at 1595.4, 1460.9 and 1319.0 cm^{-1} (Fig. 2) disappear after encapsulation indicating some modification and/or interaction between the extract and microgel carrier. (Knaul et al.,1999).

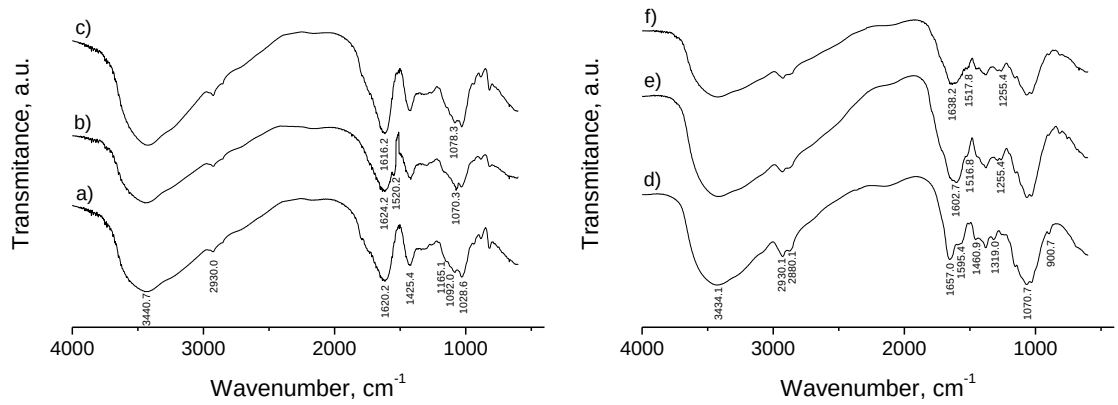


Figure 2. FT-IR spectra of a) pure alginate-chitosan microbeads, b) alginate-chitosan microbeads after extract encapsulation, c) alginate-chitosan microbeads after extract release; d) pure chitosan microbeads, e) chitosan microbeads after extract encapsulation, f) chitosan microbeads after extract release.

The observed results showed that there could be a particular arrangement within the microbeads due to the interactions of the tea polyphenolic compounds with hydroxyl and amino groups in chitosan matrix. Therefore, it is evident that addition of *Thymus serpyllum L*. extract, polyphenols could form some bonding and thus occupy the functional group of chitosan matrix.

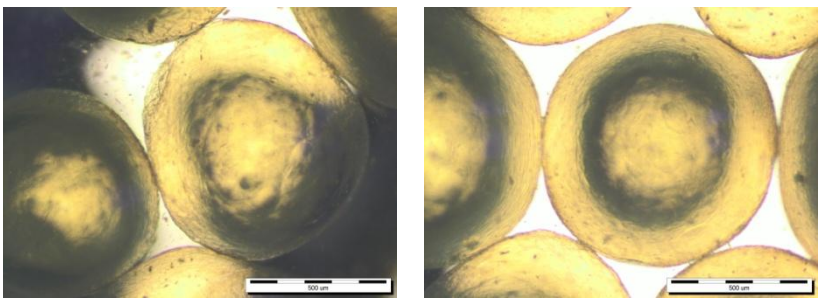
Morphology of the alginate-chitosan and chitosan microbeads

The morphologies of prepared alginate-chitosan and chitosan microbeads, dry and dispersed in distilled water, were assessed via optical microscopy.

As presented in Fig. 3, alginate-chitosan microbeads encapsulating thyme extract (Fig. 3a), as well as after extract release (Fig. 3b), were found to be spherical and discrete with the average diameter between 818 and 892 μm (Table 3), having smoother surfaces when compared to related chitosan microbeads. On the other hand, dry chitosan microbeads were smaller and had irregular shape (Fig. 2c), while after reemersion into water the sphericity was improved (Fig. 3d and 3e) and the volume increased by a factor of 3.

Table 3. Microbeads size (mean ± SD, n=3)

Formulation	Mean diameter, μm
Alginate-chitosan microbeads encapsulating thyme extract	891.5 ± 14.5
Alginate-chitosan microbeads after extract release	817.8 ± 32.1
Chitosan microbeads encapsulating thyme extract	392.2 ± 27.7
Chitosan microbeads after extract release	339.5 ± 27.4



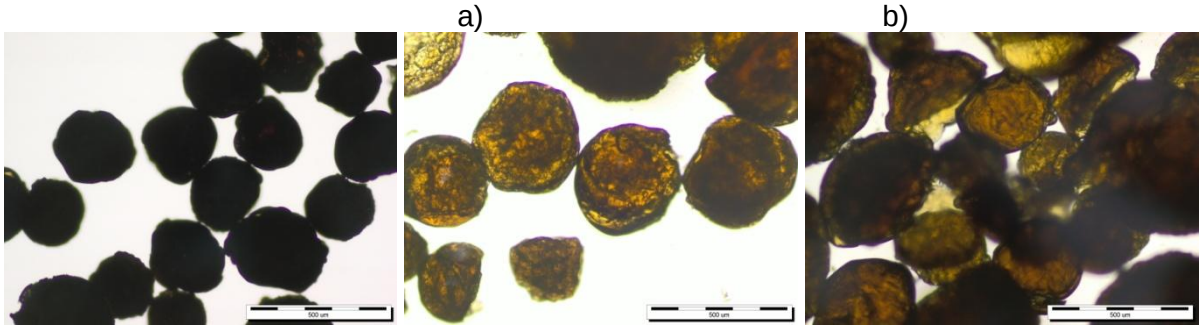


Figure 3. Optical microscopy photographs of the a) alginate-chitosan microbeads after extract encapsulation; b) alginate-chitosan microbeads after extract release; c) dry chitosan microbeads; d) chitosan microbeads after extract encapsulation and e) chitosan microbeads after extract release.

Both techniques are effective for producing microbeads of desired diameter. By increasing the applied electric potential in case of electrostatic extrusion or speed of stirring in case of emulsification (with adjustment of other operating parameters) it is possible to reduce particle size. However, a compromise regarding size of the beads has to be achieved concerning two opposite demands. Namely, larger-sized beads are necessary for a prolonged release of the encapsulated compounds while smaller-sized beads are desirable from the aspect of textural and sensorial properties of food products.

CONCLUSION

As shown in the results, both alginate-chitosan and chitosan microbeads can be used for encapsulation of polyphenolic compounds. Stability tests regarding preservation of antioxidant activity of encapsulated compounds are necessary if dosage forms developed here are going to be used in real food systems. In order to achieve desirable properties, future investigations are planned based on the matrix optimization and determination of microbeads size as well as concentration of polyphenols.

ACKNOWLEDGEMENTS

This work is supported by Ministry of Education and Science, Republic of Serbia (Project No. III46010, as well as Project No. 172062, 2011-2014).

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FERMENTED DAIRY BEVERAGES PRODUCED BY DIFFERENT STARTER CULTURES AND TRANSGLUTAMINASE

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ABSTRACT: The objective of this study was to investigate the effect of different starter cultures and transglutaminase on chemical characteristics and textural properties of fermented dairy beverages produced from milk of 0.9 $g100g^{-1}$ fat content. Samples were produced by application of a probiotic starter culture or a microfiltrated kombucha inoculum. The quality of fermented dairy beverages was analyzed after production. It was found that, two times faster fermentation was achieved in the probiotic yoghurt compared to samples of fermented milk with microfiltrated kombuha inoculum. Chemical analyses of the obtained beverages has shown that the fermented dairy beverages produced with the microfiltrated kombucha inoculum contained higher concentration of a lactose and lower level of a galactose and L-lactic acid than the probiotic yoghurt**.** Probiotic yoghurt had better textural properties than a sample with the microfiltrated kombuha inoculum. Application of a transglutaminase in a production of fermented dairy beverages improved physicochemical properties.
Key words: *fermented dairy beverages, probiotic starter culture, kombucha, transglutaminase*

INTRODUCTION

Yoghurt is a protein network formed by casein micelles entrapping serum and fat globules. Metabolic activities of the starter culture during a gelation process of milk are of specific importance. Type and ratio of microorganisms in starter culture contribute to different physicochemical and sensory characteristics of fermented dairy products. Nowadays, application of probiotics is very popular in yoghurt thanks to their prophylactic and therapeutic properties to human health (Tamime and Robinson, 2004; Tamime, 2006). Symbiotic association of yeasts and acetic acid bacteria in kombucha is well known. Metabolic activities of kombucha on a sweetened tea produce a pleasant sour beverage containing, in addition to ethanol and acetic acid, a number of useful compound. Their positive influence on a human health has been investigated and confirmed by a number of authors (Allen, 1998, Malbaša et al., 2008). Activity of kombucha on sucrose has been investigated and main pathways of a conversion of sucrose into numerous products have been determined (Malbaša et al., 2008). In addition to sucrose, application of any other sugar (lactose, glucose or fructose) is a possibility. For example, it was proved that the fermentation on lactose gave insignificant quantities of ethanol in a comparison to the fermentation on sucrose. There is a lack of research of kombucha's metabolic activity on milk (Malbaša et al., 2009; Milanović et al.; 2008, Iličić, 2010).
Improvement of rheological properties of low fat fermented milk products can be achieved by applying several techniques: ultrafiltration, addition of skim milk powder, by usage of stabilizers or by an addition of enzyme transglutaminase (TG) (Patocka et al., 2004; Sandoval-Castilla, 2004; Rodriguez-Nogales, 2006). Transglutaminase forms both inter- and intramolecular isopeptide bonds in and between food proteins. The casein in milk represents favorable substrate for cross-linking by TG application, due to non-globular easily accessible structure. Cross-linking of food proteins by TG can modify their solubility, hydration and heat stability, as well as their gelation, rheological, emulsifying or renneting properties. Rate of TG cross-linking depends on the macromolecular structure of each protein substrate (Lorenzen

et al., 2002; Carić et al., 2006; Bönish et al. 2007; Duraković et al., 2008; Milanović et al., 2009; Iličić, 2010).
The aim of this research was to examine physicochemical characteristics and textural properties of fermented milk beverages produced by application of probiotic starter cultures and the microfiltrated kombucha inoculum with or without addition of TG.

MATERIAL AND METHODS

Milk (0.9 g100g^{-1}fat, 3.1 g100g^{-1} proteins, and 4.54 g100g^{-1} lactose), obtained from AD IMLEK division Novi Sad Dairy (Novi Sad, Serbia), was used for the production of fermented dairy beverages (Table 1).
The probiotic lyophilised starter culture DIRECT-SET DELVO-YOG® MY-721 (*Streptococcus thermophilus, Lactobacillus acidophilus* LAFTI® L10 and *Bifidobacterium* sp. LAFTI® B94)**,** (DSM Food Specialties, Netherlands) and the microfiltrated kombucha inoculum were used for milk inoculation. This microfiltration of a kombucha inoculum was performed using apparatus that contains a tubular ceramic membrane module (200 nm), under process parameters: temperature 25°C, pressure difference at modules input and output 40 kPa and flow of 5 Lmin^{-1}. The concentration factor was 2.3.
Microbiological transglutaminase was isolated from *Streptoverticillium* strains (Ajinomoto Co. Inc., Hamburg, Germany). Transglutaminase Activa MP was activated in milk at 40°C for 2 hours, and then inactivated at 80°C for 1 minute prior to the fermentation. After cooling to an optimal temperature (42°C), chosen starter culture was added in milk. In all cases fermentation stopped when the pH 4.5 was reached. The gels were cooled to 8°C, homogenized by mixing and packed in plastic containers.

Table 1. Plan of experiments

No.	Description of fermented milk beverages	Sample
1.	Sample produced by probiotic application - probiotic yoghurt	PY
2.	Sample produced by probiotic application and by addition of 0.02 g100ml^{-1} TG	PY+TG
3.	Sample produced with 10% [v/v] of microfiltrated kombucha inoculum	MFI
4.	Sample produced with 10% [v/v] of microfiltrated kombucha inoculum and by addition of 0.02 g100ml^{-1} TG	MFI + TG

Physicochemical analysis

pH value of samples was measured by pH meter (Vario pH SET, Germany). The following physicochemical characteristics were analysed: total solids (TS) by oven drying, protein (TP) by Kjeldahl method, fat by Gerber method (Carić et al., 2000). Content of lactose, galactose and acetic acid was analysed by using an assay kit, test K-LACGAR 12/05, Megasyme, Ireland (Bergmeyer, 1988). Textural characteristics: firmness, consistency, cohesiveness and index of viscosity of fermented dairy beverages were measured by Texture Analyser TA.HDplus (Stable Micro System, Godalming, England) through a single compression test, using a back extrusion cell (A/BE) disc and an extension bar, using 5 kg load cell at 5°C.

RESULTS AND DISCUSSION

The fermentation process lasted two times longer in fermented dairy beverages with microfiltrated kombucha inoculum compared to the fermentation in the probiotic yoghurt (Figure 1). Fermentation time for the probiotic yoghurt sample with TG activation lasted 5 hours, while fermentation time for fermented milk beverages produced by addition of TG and the microfiltrated kombucha inoculum varied from 10 to 11 hours. These results are in concordance with the literature data (Lorenzen et al., 2002; Iličič et al., 2009; Milanović et al., 2009) showing increased fermentation time for yoghurt milk treated with the activated TG.

Færgemand et al. (1999) attributed this effect to a reduced availability of peptides' low molecular mass for the growth of lactic acid bacteria.

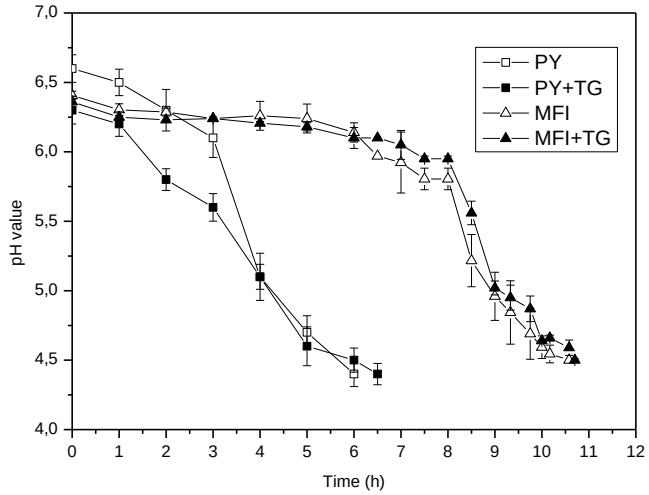

Figure 2. pH changes during milk fermentation

Table 2. Chemical composition of milk and fermented milk beverages produced by probiotic starter culture and the microfiltrated kombucha inoculum (with and without TG addition)

Samples	Characteristics ($g100g^{-1}$)				
	Total solids (TS)	**Total proteins (TP)**	**Lactose**	**Galactose**	**L-lactic acid**
Milk (M)	9.85±0.33	3.15±0.04	4.74	0.0115	0,0015
PY	9.89±0.01	3.19±0.02	3.33	0.640	0.81
PY+TG	9.72±0.08	3.30±0.06	3.26	0.640	0.869
MFI	9.63±0.26	2.78±0.05	3.89	0.557	0.535
MFI+TG	9.86±0.29	2.73±0.22	3.74	0.601	0.565

Total solids and total protein in probiotic yoghurt were higher than in kombucha fermented milk beverages. During fermentation of milk with usage of probiotic starter culture, lactose content decreased around 44%.

Lactose content of the probiotic yoghurt was approximately 16% lower compared to samples produced with the microfiltrated kombucha inoculum (Table 1). All samples had higher content of lactose compared with literature data (Belloso-Moralles and Hernandez-Sanchez, 2003). Belloso-Moralles and Hernandez-Sanchez (2003) found that samples produced from whey contained about 1 $g100g^{-1}$ of lactose after fermentation. Content of galactose and L-lactic acid in all samples was higher than in milk used for the fermentation (Table 2).

Differences in chemical composition between probiotic yoghurt and kombucha fermented milk beverages were result of different metabolic and microbiological characteristics of probiotic starter culture and microfiltrated kombucha inoculums.

Table 3. Textural properties of fermented milk beverages produced by probiotic starter culture and the microfiltrated kombucha inoculums (with and without TG addition)

Samples	Characteristics			
	Firmness (g)	Consistency (gs)	Cohesiveness (g)	Index of viscosity (gs)
PY	15.05±1.08	443±14.82	-7.637±1.6	-4.361±1.371
PY+TG	22.8±1.13	588.73±23.1	-18.79±1.23	-29.78±4.6
MFI	13.86±1.09	394.70±40.43	-5.02±0.8	-1.205±0.663
MFI+TG	21.32±7.4	555.981±187.4	-16.591±7.6	-23.962±2.4

Textural characteristics of fermented dairy beverages are presented in Table 2. Probiotic yoghurt with TG had the highest textural characteristics (firmness 22.8 g, consistency 588.73 gs, cohesivness -18.79 g, index of viscosity -29.78 gs), while the sample MFI had lowest textural characteristics (firmness 13.86 g, consistency 394.70 gs, cohesivness -5.02 g, index of viscosity -1.205 gs). This is in accordance with Iličić et al. (2009) where fermented dairy beverages containing 1.5% of the kombucha concentrated inoculum and 0.02% of activated TG had the highest textural characteristics (firmness 34 g, consistency 900 gs, cohesivness -33 g, index of viscosity -80 gs). Samples with 3% of concentrated inoculum (without TG) had the lowest textural characteristics (firmness 14 g, consistency 400 gs, cohesivness -7 g, index of viscosity -5 g) (Iličić et al., 2009).

CONCLUSIONS

The probiotic starter culture and the microfiltrated kombucha inoculum had significant influence on differences in physicochemical and textural properties of fermented dairy beverages. Fermentation process was faster in the the probiotic yoghurt compared to all fermented dairy beverages produced with the microfiltrated kombucha inoculum. Addition of TG at level of 0.02% contributed significantly to the formation of stirred fermented milk beverages with improved physical characteristics and textural properties.

ACKNOWLEDGEMENTS

Authors would like to thank the Ministry of Education and Science of the Republic of Serbia for a financial support for this study, which is a part of project III 46009. Also, authors are grateful to Ajinomoto Company for the sample of enzyme transglutaminase, as well as to NS Trading, Novi Sad for gratis sample of the probiotic starter culture.

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INVESTIGATION OF PROBIOTIC POTENTIAL OF *LEUCONOSTOC* SP. AND *LACTOBACILLUS* SP. NATURAL ISOLATES FROM NON-COMMERCIAL MILK AND WATER KEFIR

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ABSTRACT: Non-commercial milk and water kefir grains (from a household in Belgrade, Serbia) were used for isolation of lactic acid bacteria on TSA, MRS and M17 agar.
Preliminary identification has been performed according to morphological traits (Gram staining, cells shape and colony characteristics on agar plate). Isolates suspected to lactic acid bacteria were tested for selected physiological and biochemical characteristics. Investigations of potential probiotic properties included antimicrobial activity against three different pathogens, sensitivity to simulated gastrointestinal tract conditions and autoaggregation ability. In total, ten cultures were selected for further analysis, five from each milk and water kefir grains.
The morphological and physiological profiles showed that isolated strains belong to genera *Lactobacillus* and *Leuconostoc*. There were some differences in their sugar fermentation pattern as well as in their antimicrobial activities, with milk kefir cultures being more potent pathogen inhibitors. In simulated gastrointestinal tract conditions all strains showed high sensitivity towards bile acids, while their growth in low pH and pepsin was good. Two cultures only (one from water kefir grains and one from milk kefir grains) showed good autoaggregation ability.
Overall, most isolates exposed good probiotic potential. There are no significant differences between lactic acid bacteria isolated from milk and water kefir grains.
Key words: *lactic acid bacteria, probiotic, milk and water kefir*

INTRODUCTION

Functional foods remain the hottest topic in the food industry and nutrition. The term functional foods means products with a potential positive effect on health. This food beside basic food nutrients (fats, proteins, carbohydrates) contain specific biologically active substances that have a proven positive effect on human health and that may be useful in the treatment of many chronic diseases (different types of cancer, high cholesterol, etc.), (Annuk et al., 2001).
Food products containing probiotic microorganisms are especially highlights of numerous researches (Vaughan and Mollet, 1999). There are numerous studies about the positive physiological and health effects after regular intake of probiotics on human health, the prevention of acute and chronic diseases, work capacity, etc (Fuller, 1989).
Kefir is fermented beverage produced by the action of lactic acid bacteria, yeasts and acetic acid bacteria on milk. The microbiological population is firmly embedded in kefir grains. The grains have been described as resembling elastic small florets similar to cauliflower in shape, yellow or white in color, and 20-30 mm in size (Farnworth, 2008). However, there is another beverage, made from sugar and water with figs and lemon added, whose fermentation is induced by grains of a different type which are transparent, mucilaginous, but less resilient. The grains of the water kefir are described to consist of dextran, an α 1-6 linked glucose polymer in contrast to milk kefir grains,which are made of more complex heteropolysaccharides, namely kefiran. Microbiota of water kefir grains also consists of LAB and yeasts (Pidoux, 1989).

Beneficial effects of milk and water kefir on human health are anticipated by consumers but have not yet been scientifically substantiated. Nevertheless, probiotic properties are widely spread among lactic acid bacteria (LAB).
Probiotic properties of LAB are proven in numerous clinical and laboratory studies. It is primarily reflected in the good antimicrobial activity and ability to survive adverse conditions in the gastro-intestinal tract (GIT) (Macfarlane and Cummings, 2002, Kaur et al., 2002).
The aim of this work is to characterize lactic acid bacteria isolated from non-commercial milk and water kefir according to the requirements for probiotics in order to consider their further application in the development of new functional products.

MATERIALS AND METHODS

Isolation and phenotypical characterization of non-commercial milk and water kefir isolates

Non-commercial milk and water kefir grains (from a household in Belgrade, Serbia) were used for isolation of lactic acid bacteria. The grains were mechanically homogenized and appropriate dilutions were inoculated into sterile Petri dishes with 3 different media: TSA (Tryptic Soy Agar), MRS and M17 agar. After 2 days incubation at 30°C, five to ten colonies from each sample were inoculated in MRS broth and then stored at -20 °C in MRS broth containing 20% glycerol. Strains were routinely propagated in MRS broth at 30°C in microaerofilic condition.
The first tentative identification as LAB was performed according to morphological characteristics (cells shape, Gram staining) and physiological characteristics (catalase test, growth at different temperatures, growth in milk and litmus milk, growth in media with different concentration of NaCl and sugar fermentation).

Antimicrobial activity

Selected isolates were screened for their ability to inhibit strains: *S. aureus* ATCC 25923, *C. albicans* ATCC 10259 and *E. coli* ATCC 25922. This was performed by using the spot method where 2 µl of active (overnight) MRS culture of isolates were spotted on TSA. Plates were dried for 30 min at room temperature and incubated semi anaerobically at 30°C for 18 h; overlaid with 6ml of TSB containing 0.6% agar at 45°C, seeded with 1% (v/v) of an active overnight culture of the target strain and incubated aerobically at 30°C for 18 h.
Sensitivity to simulated gastrointestinal conditions (gastric test and bile salts test)

Gastric test

0.5 % NaCl and 0.3% pepsin solution is simulating gastrointestinal conditions. pH of this solution is set to 3.2 with 1M HCl and solution is filtered sterile. 9 ml of this solution was inoculated with 1 ml overnight culture of selected strains and was incubated at 30°C aerobically. After 2h cell count was determined on agar plates (TSA). The plates were incubated aerobically at 30°C for 24h. The percentage of survived cells was determined by comparing the number of colonies from gastric juice and that from saline which has been used as a control. Each strain was tested twice.

Bile salts test

MRS broth with 0.05% and 0.1% bile salts (Oxgall) were simulating duodenal conditions. 3 ml of this solution was inoculated with 50 µl overnight cell culture and incubated on 30^{o}C. After 24h cell concentration was determined by optical density (620 nm). The percentage of survived cells was determined by comparing the cell concentration in MRS broth with bile salts and MRS broth without bile salts which has been used as a control. Each strain was tested twice.

Autoaggregation ability

The cell cultures were grown 18 hours at 30ºC and centrifuged at 7000 rpm for 15 minutes. Supernatant was drained; the cells were washed twice and resuspended in saline. 4 ml of suspension was than mixed in a vortex at maximum speed and the degree of autoaggregation was determined during the next 5 hours at room temperature. For every hour 0.1 ml of suspension was taken from the top, poured in 3.9 ml saline, and mixed. Optical density was measured on colorimeter using a yellow filter (570 nm). Autoaggregation percentage is calculated by the following equation:

% autoaggregation = 1- (At / Ao)* 100

Ao – absorbance measured at t = 0h

At – absorbance measured every hour

RESULTS AND DISCUSSION

In the present study, 10 bacteria were isolated from milk and water kefir grains. Strains isolated from milk kefir are signed as K1 to K5, and those from water kefir are signed as T1 to T5. Isolated bacteria were able to grow on MRS, M17 and TSA plates at 30°C. All strains tested were catalase-negative and Gram-positive with various shapes ranging from long rods to coccoid rods. Testing the growth at different temperatures showed that optimal temperature is 30°C for all strains, all can grow at 37°C and only one (T1) cannot grow at 15°C. Nine strains were tolerant to NaCl at concentrations of 4% and 6.5%, but not at 9.6%; only T1 did not grow in the presence of NaCl at all. The absence of gas production from glucose was used to differentiate homo- and heterofermentative group. Sugar fermentation was used for determination of fermentation pattern of isolates. The results of physiological characteristics are shown in Table 1 and sugar fermentation patterns are presented in Table 2. According to these results 4 of isolated species belong to genus *Lactobacillus* and 6 to genus *Leuconostoc*.

Table 1. Physiological characteristics of isolated strains

Isolates	Catalase	Growth at different temperatures (°C)			Growth in the present of different concentrations of NaCl (%)			Fermentation		Coagulation of skim milk/pH	Growth in litmus milk/pH	EPS production
		15	30	37	4	6.5	9.6	Glu/ gas	Gln/ gas			
K1	-	+	+	+	+/-	+/-	+/-	+/-	-/-	- /6.42	R/6.53	-
K2	-	+	+	+	+	+/-	+/-	+/+	-/-	C /4.25	ACR/4.30	+
K3	-	+	+	+	++	+	-	+/+	+/+	C /4.36	ACR/4.30	+
K4	-	+	+	+	++	+	-	+/+	+/+	- /6.10	R/5.91	-
K5	-	+	+	+	++	+	-	+/+	+/+	- /6.64	R/6.74	+
T1	-	-	+	+	-	+/-	+/-	+/+	-/-	C /4.80	R/5.04	+
T2	-	+	+	+	+/-	+/-	+/-	±/+	-/-	- /6.37	R/6.52	-
T3	-	+	+	+	++	+	+/-	+/+	+/+	- /6.40	R/6.61	+
T4	-	+	+	+	+/-	-	-	+/+	-/-	C /4.85	ACR/4.90	+
T5	-	+/-	+	+	+/-	+/-	+/-	+/-	-/-	- /6.51	R/6.76	+

Glu – glucose, Gln - gluconate

Many LAB strains are known by exopolysaccharides (EPS) production (Ruas-Madiedo & de los Reyes-Gavilán, 2005). This property was used for characterization of isolates. They formed mucous colonies on modified MRS agar (with sucrose instead of glucose) which is indication of EPS production (Table 1).

Table 2. Sugar fermentation pattern after 7 days

Substrates		Isolates									
		K1	K2	K3	K4	K5	T1	T2	T3	T4	T5
DARA	D-arabinose	++	-	+	++	-	-	+/-	-	-	-
RIB	Ribose	-	-	-	+/-	-	-	-	+	-	++
DXYL	D-Xylose	-	++	++	++	++	-	+/-	++	-	++
GAL	Galactose	++	++	++	++	++	++	++	+/-	++	+
GLU	Glucose	++	++	++	++	++	++	+/-	++	++	++
MNE	Mannose	++	++	++	++	++	++	++	++	++	++
MAN	Mannitol	-	-	++	+/-	++	-	++	+/-	-	+
SOR	Sorbitol	-	-	-	++	-	+	-	+/-	-	-
ESC	Esculin	++	++	++	++	++	++	++	++	++	++
SAL	Salicin	++	++	++	++	++	++	++	-	++	-
CEL	D-cellobiose	-	++	++	+/-	++	++	+/-	++	++	++
MAL	D-maltose	++	++	++	++	++	++	+/-	++	++	++
LAC	D-lactose	++	++	++	++	++	++	+/-	+	++	+
MEL	D-melibiose	++	-	-	+	-	++	+/-	+/-	-	++
SAC	D-sucrose	++	-	++	++	++	+	+/-	++	++	++
TRE	D-trehalose	++	++	++	++	++	-	+/-	++	-	++
RAF	D-raffinose	-	-	-/+	++	-	+	+/-	-	+/-	+
GNT	Na-gluconate	-	-	++	++	++	-	-	+	-	+

++ yellow; + green; +/- brownish green; - no change of color

The antimicrobial activities of the LAB strains tested were variable (Table 3).
According to the presented results it can be seen that all strains showed a certain antimicrobial activity under the given conditions of the experiment. Isolates from milk kefir are more potent pathogen inhibitors.

Table 3. Antagonistic activity of kefir isolates against the pathogen indicators

Pathogen strains			**Diameter of inhibition halo* (mm)**									
			Isolates									
			K1	K2	K3	K4	K5	T1	T2	T3	T4	T5
S. aureus	ATCC	25923	20	26	25	ND	28	8	8	26	12	7
E. coli	ATCC	25922	16	20	25	ND	27	10	8	30	13	0
C.albicans	ATCC	10259	18	26	23	23	26	10	8	ND	19	0

ND - not determined

Acid tolerance is a property that any strain expected to have effects in the GIT should possess (Salminen et al., 1996, Marteau et al., 1997). Resistance at pH 3.2 for 2h (Arihara et al., 1998) is one of the standards for low pH tolerance of probiotic bacteria. All strains showed high survival (100%) at low pH, with two strains from milk kefir had a slightly lower percentage with about 60 - 70%, (Table 4).

Table 4.
Sensitivity to simulated GIT conditions

Table 5.
Autoaggregation ability

Isolate	Percent of survived cells		
	HCl, pepsin	Bile acids	
	2h	0.05%	0.1%
K1	58.75	15.8	0.0
K2	70.77	14.3	0.0
K3	92.45	13.3	3.3
K4	104.85	8.6	0.0
K5	109.28	21.4	0.0
T1	89.50	20.5	2,1
T2	94.27	21,8	2,3
T3	98.88	21.7	4.3
T4	93.97	26.1	4.3
T5	103.08	25.0	6.25

Isolate	% of autoaggregation after specified time (hours)				
	1	2	3	4	5
K1	0.00	16.67	50.00	50.00	50.00
K2	50.00	50.00	50.00	50.00	50.00
K3	33.33	33.33	33.33	33.33	66.67
K4	63.64	100.00	100.00	100.00	100.00
K5	0.00	40.00	20.00	20.00	20.00
T1	16.67	50.00	66.67	66.67	66.67
T2	0.00	16.67	16.67	16.67	33.33
T3	33.33	16.67	33.33	50.00	50.00
T4	0.00	0.00	0.00	0.00	0.00
T5	0.00	16.67	16.67	33.33	33.33

The results indicate that the strains could survive in the acidic stomach environment and reach the areas of beneficial activity (small intestine and colon) when ingested.
After passage through acidic stomach conditions it is important that, for application of LAB strains as probiotics, they are able to survive the bile salt in the intestine. Most researchers assess bile resistance within the range of 0.1-0.5% (Lankaputhra and Shah, 1995). At concentration of 0.1% viability was very low for all strains. Better results were observed at bile concentration of 0.05%, although the percentage of survival is low. Strains isolated from water kefir showed better tolerance to the conditions in the intestine (Table 4). The probably reason for poor surviving is that the isolated strains are not of intestinal origin.
Autoaggregation ability points to the possibility of cell attachment to one another as well as the ability to form biofilms on the walls of the intestinal epithelium. It can affect the ability to retain in their communities as well as resistance to the effects of adverse conditions (Vandevoorde et al., 1992, Boris et al., 1997). Two cultures only (K4 from water kefir grains and T1 from milk kefir grains) showed good autoaggregation ability (Table 5).

CONCLUSIONS

There are lots of problems in reliable characterization of probiotic strains using *in vitro* methods, but the initial screening of strains in this manner remains useful for detection of probiotic candidates.
Determination of fermentation pattern of lactic acid bacteria is the first step in the identification of strains, but molecular methods are needed for accurate determination.
More research is needed on other potential probiotic properties of the strains such as antibiotic resistance, antimicrobial activity against increased number of pathogen strains and ability to adhere and colonize human intestinal cells.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31035 “Application of biotechnological methods for sustainable exploitation of by-products of agro-industry” financed by the Ministry of Science and Technological Development, Republic of Serbia.

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MICROENCAPSULATION OF POTENTIAL PROBIOTIC STRAIN *LACTOBACILLUS PLANTARUM* JS7A BY SPRAY DRYING

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ABSTRACT: In the present study, we investigated the use of spray drying as a way to prepare dairy-based powders with high number of viable cells of the potential probiotic strain *L. plantarum* JS7A. Microencapsulation was done using reconstituted skim milk (RSM), inulin and maltodextrin as carriers. When RSM was used as a spray drying carrier a microbial survival rate of 73 % was achieved, although the partial substitution of skim milk with inulin also resulted in high level of survival of 70 %. The poorest survival of 41 % was obtained with maltodextrin as a carrier. The moisture contents in powders were ranging from 4.1 % to 4.4 %. Spray drying process stressed the cells which was shown by increased sensitivity to NaCl. However, it was obtained that reconstituted skim milk showed the significantly lower inhibition of cells of 49 % comparing with inulin and maltodextrin with 55 and 75 % of inhibition, respectively.
Key words*: microencapsulation, potential probiotics, Lactobacillus plantarum, spray drying*

INTRODUCTION

Probiotics are live microorganisms which when administered in adequate amounts, confer a health benefit on the host (FAO, 2006). However, many studies have demonstrated their poor viability and stability in the fermented products. The different techniques of encapsulation, which include formation of a protective layer around the bacterial cells, can be applied for their better survival under adverse environment (Zuidam and Nedović, 2010).
There are a several techniques available for the production of encapsulated probiotics as follows: extrusion, emulsion, drying (fluidized bed, freeze, spray) and spray-coating technique (Petrović et al., 2007; Manojlović et al., 2010). Spray drying is an economical process of preservation of probiotic cultures, which provides high productivity and relatively low operating costs. Spray drying is a methods when an active material is dissolved or suspended in a melt or polymer solution and became trapped in the dried particles.The main advantages of spray drying is the ability to handle heat sensitive materials such as protein, enzymes, flavors, living cells etc., because of the short contact time in the dryer.
Reconstituted skim milk is the most common used carrier for spray drying of probiotics, because it is widely used in the dairy industry (Desmond et al., 2002; Corcoran et al., 2004; Ananta et al., 2005). Furthermore, milk proteins can form a protective layer around the bacterial cells, while calcium enhances cell survival after dehydration. The prebiotics like inulin could be also used as a carrier, for enhance the viability of probiotic cultures in the gastrointestinal tract (Corcoran et al., 2004).
In this study the possibility of using spray drying technique for microencapsulation of potential probiotic *Lactobacillus plantarum* JS7A, with different carriers, reconstituted skim milk, prebiotics inulin and carbohydrate maltodextrin was investigated (Petrović, 2011). Also, the degree of cell damage as a result of high temperature of drying was determined.

MATERIAL AND METHODS

Bacterial strain and culture condition

Potential probiotic strain JS7A which was isolated previously from the fermented carrot and was identified as member of *Lactobacillus plantarum,* by PCR based methods (Petrović, 2011) was obtained from the strains collection of the Department of Industrial Microbiology, Faculty of Agriculture, Belgrade.

Fresh 24 hours culture (1% v/v) was inoculated in MRS broth and incubated for 20 h at 37 °C in order to achieve stationary phase of growth. For spray-drying purposes strain was cultured as follows. Cells were harvested by centrifugation (4 500 x g, 15 min) from the incubated broth and rinsed in saline solution. The resulting culture was then used to inoculate the feed media.

Spray-dried feed media

Spray-dried feed media was consist of either reconstituted skim milk - RSM (20 % w/v), RSM (10 % w/v) and maltodextrins (10 % w/v) or RSM (10 % w/v) and inulin (10 % w/v). Feed media were prepared by dissolving the carrier in distilled water and their sterilization by autoclaving at 105 ° C, 5 min. For the adaptation of LAB to the drying temperature, inoculated media were incubated at 37 °C, for 30 min.

Spray drying of potential probiotic strain

A laboratory scale spray dryer (model B290 Büchi mini spray dryer, Switzerland) was used to process samples at a constant air inlet temperature of 170 ºC. The feed solution was atomized in drying chamber using a two-fluid nozzle. The outlet air temperature was maintained at 80 - 85 ºC, in order to obtain powders with around 4% (w/w) moisture (Master, 1991).

Per cent of survived bacteria were calculated as follows:

$$\% \text{ survivors} = N/N_0 x100,$$

where N_0 - represented the number of bacteria in medium before drying and N - was the number of bacteria in spray-dried powder. Both N and N_o were expressed per gram of dry matter.

Salt tolerance test

The sensitivity of tested cultures to NaCl before and after drying was determined as described by Gardiner *et al.* 2000. Fresh overnight MRS broth culture and culture-containing spray-dried powders were pour plated onto MRS agar supplemented with 5% NaCl. The plates were examined after 72 h of anaerobic incubation at 37 °C, and the colony sizes and numbers were compared with the colony sizes and numbers on MRS plates without NaCl.

The percent of inhibition of bacterial cells in medium supplemented with 5 % of NaCl, was calculated as followed:

$\% I = N_0 - N / N_0$, where:

N_0 - represented the colony numbers from the fresh overnight culture inoculated in MRS with 5 % of NaCl;

N – represented the colony numbers of spray-dried bacteria inoculated in MRS with 5 % of NaCl.

Moisture content in spray-dried powders

The moisture content in the spray-dried powder was determined by drying 3 - 5 g of powder in the oven at 102 ± 2 °C. On the basis of differences in the sample weight before and after drying it was calculated the moisture content (%).

RESULTS AND DISCUSSION

In order to obtained spray-dried powders with high numbers of potential probiotic strain *L. plantarum* JS7A, it must withstand the high temperatures encountered during spray-drying, which has been shown to vary among strains of probiotic lactobacilli (Gardiner et al. 2000). Also, the viability of lactobacilli during spray drying depends on the growth phase of bacteria prior to drying. Teixeira et al. (1994) have previously shown that exponential - phase cells of *L. bulgaricus* are more susceptible to spray drying than cells in the stationary phase of growth. From this reason the tested culture was grown to stacionar phase prior to spray drying, which may have increased their survival to the process.
Regarding the total solids content of spray-drying medium (RSM, and combination of RSM with inulin and maltodextrin) it was held constant at 20 % (w/v), because the solid content of 20 % has been consider as optimum for assuring high residual viability of different strains of lactic acid bacteria (Desmond *et al.* 2001; Gardiner *et al.* 2000; Mauriello *et al.*1999; Johnson i Etzel, 1993).
In addition, the outlet air temperature of 80 to 85°C was held constant during drying as it was necessary in order to obtain powders with moisture contents that did not exceed the level required for prolonged powder storage life (Master, 1991). In this work the moisture content in powders were ranging from 4.1 % to 4.4 % which was regarded as a good-quality parameter of dried-dairy products.
The figure 1 shows a very good survival of *L. plantarum* JS7A in powder with 20 % RSM of 73 % (with $2.77x10^{10}$ CFU g^{-1} of viable cells) while in combination of RSM with inulin it was 70 % of survived bacteria. In the combination of RSM with maltodextrin the significantly lower survival of *L. plantarum* JS7A of 41 % was obtained.. On the other hand, in the work of Ananta et al., (2004) it was obtained the good survival of probiotics using a combination of carriers as maltodextrin, gelatin, glycerol, and lactose.

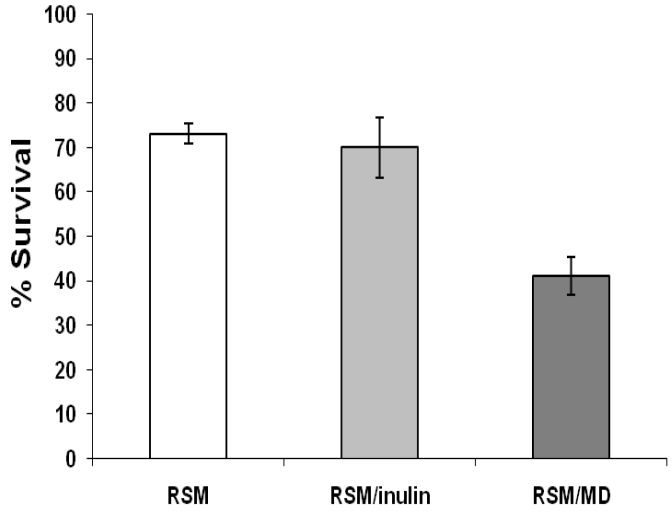

Figure 1. Average (± standard deviation) survival of *L. plantarum* JS7A spray-dried in the presence of reconstituted skim milk (RSM) (20 % w/v), RSM (10 % w/v) and inulin (10 % w/v) and RSM (10 % w/v) and maltodextrin (10 % w/v).

In addition, after spray drying at outlet temperature of 70 - 75 °C, it was obtained high survival rate of 97 % for *L. paracasei* NFBC 338 in 20 % RSM, while significantly lower survival of only 11 % was performed for *L. salivarius* UCC 18 at lower outlet temperature of 60 - 65 °C (Gardiner et al., 2000).
A potential disadvantage of spray-drying process as a way to preserve microbial cultures is the damage on bacterial cells caused by high temperature. In order to study potential cellular damage resulting from the spray-drying process the sensitivity of tested cultures to NaCl before and after drying as described by Gardiner *et al.* 2000 was determined. Increased

sensitivity of sublethally injured bacteria to NaCl has been associated with cytoplasmic mambrane damage (Brennan et al. 1987; Teixeira *et al.* 1995a).
Spray dried *L. plantarum* JS7A exibited the highest damages with approx. 75 % of inhibition in the presence of NaCl in the RSM/MD mixture while it was 55% and 49% in RSM/inulin and RSM feed respectively (Figure 2). When spray-dried powder was plated in the presence of 5% NaCl, colony size was found to be considerably reduced compared to the colony size prior to spray drying (data not shown). This morphological change, in the presence of NaCl, indicates that the spray drying process stressed the cells.

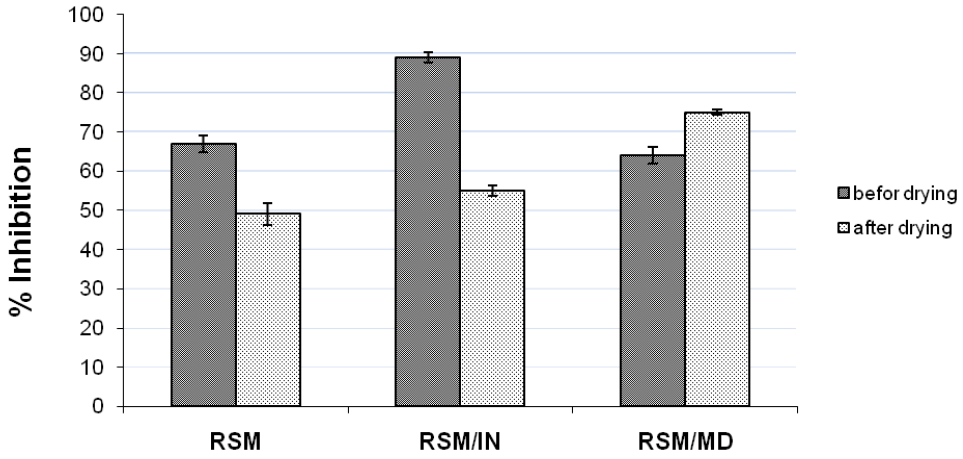

Figure 2. Average (± standard deviation) percent of inhibition of spray-dried *L. plantarum* JS7A cells with high concentration of NaCl

The high inhibition by NaCl was obtained for *L. salivari*us UUC 500 with 90 % susceptibility to NaCl, after drying (Corcoran *et al*. 2004). In addition, Gardiner *et al.* (2000) has been reported the total inhibition (100 % of damaged cells) with 5 % NaCl for *L. salivarius* UUC118 in RSM feed.
It can be generally concluded that probiotic survival is dependent on the spray-drying carrier applied, as differences in thermal conductivity and diffusivity can affect survival of spray-dried probiotic (Lian et al. 2002). However, the partial substitution of RSM with carbohydrate maltodextrin in the feed solution during drying demonstrated the poorest survival and protection of tested culture compared with RSM as a whole carrier.

CONCLUSION

Our data demonstrate that spray drying may be a cost-effective way to produce large quantities of some probiotic culture. Also, powder obtained with skim milk or inulin as a carrier could be a useful ingredients in functional food applications.

ACKNOWLEDGMENTS

This work was supported by Ministry of Education and Science, Republic of Serbia (Grant No 46010).

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MICROENCAPSULATION OF NATURAL ANTIOXIDANTS FROM *PTEROSPARTUM TRIDENTATUM* IN DIFFERENT ALGINATE AND INULIN SYSTEMS

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ABSTRACT: The bioactivity of natural antioxidants from plant extracts is well known. Still, the effectiveness of these natural antioxidants, namely polyphenols, depends on preserving their stability, which can be increased by microencapsulation. The aim of this study was to protect natural antioxidants from the aqueous extract of Portuguese wild herb *Pterospartum tridentatum* by encapsulation in alginate hydrogel microbeads. Microbeads were prepared by electrostatic extrusion technique: plain Ca-alginate microbeads and Ca-alginate microbeads with 10 and 20 mass% of inulin as a filler substance. Total polyphenol content (TPC) and the radical scavenging activity using ABTS and DPPH cations were determined. The release studies of polyphenols from microbeads were performed. The microbeads were analysed by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and optical microscopy (OM). Encapsulation efficiency (EE) was in the range from 49 to 73%. Antioxidant assays and release studies showed that alginate-inulin microbeads appeared to be suitable dosage forms. The inclusion of inulin contributes to improved microbeads structure, as well as to nutritional values of food. Thereby, potential applications of these microbeads could be functional food products, an increasingly valued market.
Key words: *encapsulation, electrostatic extrusion, antioxidants, Pterospartum tridentatum, alginate*

INTRODUCTION

From the ancient times, herbs and spices were being added to different food and beverages to improve flavour. It has been also shown that bioactive compounds commonly found in herbs contain health benefits because of their significant antimicrobial and antioxidant capacities and anticarcinogeni activities (Cao et al., 1999). Various bioactive compounds, such as alkaloids and flavonoids, have been identified in aqueous extracts of *Pterospartum tridentatum* which is wild herb widely used in traditional medicine and cuisine (Vitor et al., 2004). *Pterospartum tridentatum* L. is a European endemic species belonging to the subfamily *Papilionoideae* (Talavera, 1999) and known as *carqueija* or *carqueja* in Portugal. Some authors refer the use of this herb in popular medicine for colds, stomach aches, intestinal problems, kidney disease, liver and bladder problem. However, most of bioactive compounds are very sensitive to many factors. The effectiveness of these natural antioxidants, namely polyphenols, depends on preserving their stability, which can be increased by microencapsulation.

Microencapsulation is an effective method to protect bioactive components, preserve their stability during processing and storage and prevent undesirable interactions with food matrix (Wandrey et al., 2009, Nedović et al., 2011). Electrostatic extrusion is technique used for encapsulation of the compounds by production of spherical, small and uniform microparticles which are desirable in food applications (Manojlović et al., 2008, Kostić et al., 2012).

The goal of the present study was to develop *P. tridentatum* extract formulations aimed at delivery of bioactive compounds in functional food products. The extract was encapsulated

in alginate and alginate-inulin microbeads by electrostatic extrusion and the obtained microbeads were characterized from the aspect of TPC and antioxidant activity. Besides hydrogel microbeads, freeze-dried forms of microbeads were also assessed, as they are convenient for long-term applications.

MATERIAL AND METHODS

Na-alginate (medium viscosity) was purchased from Sigma. Folin-Ciocalteu, Na-carbonate, Ca-chloride and Na-citrate were of analytical grade and supplied by Sigma-Aldrich (St. Louis, MO, USA). 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium chloride, potassium persulfate were obtained from Sigma -Aldrich (Germany). Inulin was generously gifted from a local milk factory.

Preparation of microbeads

The appropriate amount of *P. tridentatum* aqueous extract (2 mg mL^{-1}) was added to Na-alginate water solution (1,5% w/v) to prepare extract-alginate solution. In addition, extract-alginate solution was mixed with 10 and 20 mass% of inulin to prepare extract-alginate-inulin solutions. Both types of solutions were submerged to electrostatic droplet generation in order to produce alginate and alginate-inulin microbeads entrapping extract compounds as described in the next paragraph.

The obtained solutions were extruded through a blunt stainless steel needle (23 G) at a constant flow rate of 25,2 mL h^{-1}, by a syringe pump (Razel Scientific Instruments, Stamford, CT, USA). The extrusion was performed under an applied electric field between the positively charged needle and grounded collecting solution. The potential difference was controlled by a high voltage unit (Model 30R; Bertan Associates, Inc., New York, USA) and kept at a constant voltage of 7,0 kV. Collecting solution was mixture of *P.tridentatum* extract and Ca-chloride 1,5% (w/v). After ions exchange, alginate droplets formed insoluble hydrogel microbeads with the extract entrapped in. The microbeads were left in the cross-linking solution for 30 min and then used for further analysis (Bugarski et al., 2004). Hydrogel microbeads were observed under optical microscope (Olympus CX41RF, Tokyo, Japan) and average diameter was measured with the image analysis program Cell^{A} (Olympus, Tokyo, Japan).

In order to produce freeze-dried beads, hydrogel microbeads were frozen at −80°C for 1 h before freeze drying, which was carried out at −50°C at a pressure of 30 Pa for 10 min and 10 Pa for 24 h. The surface morphology of carefully freeze-dried microbeads was determined by scanning electron microscopy (SEM, model RESCAN MIRA3XMU, Czech Republic).

Fourier transform infrared spectroscopy (FTIR)

The interactions between the different components of the alginate-inulin systems were analysed by Fourier transform infrared (FTIR) using a FTIR spectrophotometer (BOMEM, Hartmann & Braun). Microbeads samples were dried in a vacuum desiccator, triturated with micronized KBr powder and compressed into discs by pressing the powders. Discs and scanned from 4000 to 450 cm^{-1} at a resolution of 4 cm^{-1} at room temperature.

Determination of total phenol content (TPC)

TPC was determined using the Folin-Ciocalteau reagent, according to a modified method of Lachman et al (1998). In brief, 70 µL of the sample was pipetted into a 15 mL volumetric flask containing 350 µL of Folin–Ciocalteu reagent, 4,2 mL of distilled water and 1,05 mL of 20% (w/v) Na-carbonate, and the volume was made up with distilled water. After 2h, the absorbance of was measured at 765 nm against a blank sample. Gallic acid was used as the standard and the results expressed as mg L^{-1} of gallic acid equivalents (GAE).

Encapsulation efficiency

Encapsulation efficiency (EE%) was calculated as the amount of TPC encapsulated in microbeads (m_b) divided by the TPC of the solution used for the preparation of microbeads (m_s), as shown in equation 1:

$$EE\% = m_b/m_s \times 100 \quad (1)$$

Quantification of TPC in microbeads (m_b) was performed after dissolving microbeads in 2% (w/v) Na-citrate solution (in a weight ratio of 1:5), using a Vortex mixer to chemically dissolve them at room temperature. TPC was determined using the Folin–Ciocalteu method according to the procedure described in the upper section.

Determination of free radical-scavenging ability

The radical scavenging activity of the extract was determined according to Dudonne et al. (2009). The DPPH• solution in ethanol ($6x10^{-5}$M) was mixed with 100 µL sample and after 30 min the decrease in absorbance at 515 nm was measured (A_E). A blank sample contained 100 µL of ethanol (A_B). Percentage of cation inhibition was calculated using equation 2:

$$\% \text{ inhibition} = [(A_B - A_E)/A_B] \times 100 \quad (2)$$

where (A_B) is absorbance of the blank sample and (A_E) is absorbance of the sample.

The free radical scavenging capacity of the extracts was also studied using the ABTS radical assay. ABTS• was produced according to Re et al. (1999). An appropriate solvent blank reading was taken (A_B). Extract solutions (30 µL) was mixed with 3 mL of ABTS• solution and absorbance reading was taken after 6 min (A_E). The percentage of inhibition of ABTS• was calculated using equation 2.

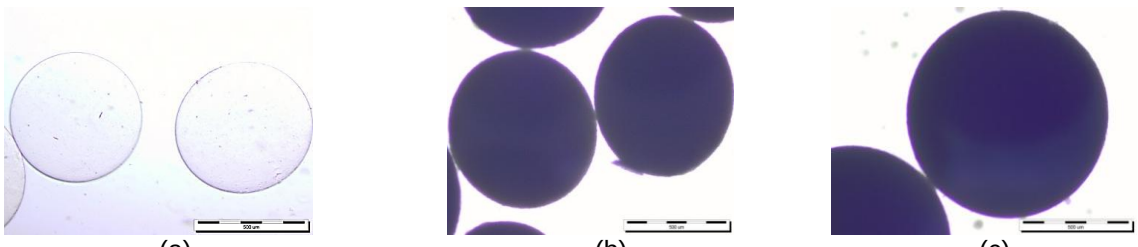
In order to test radical scavenging activity of the extract after microencapsulation, certain amount of hydrogel microbeads with encapsulated extract was suspended in 3 mL of distilled water. The samples were left on an orbital shaker operating at 100 rpm and when the extract was completely released the samples were analysed on TPC, DPPH and ABTS.

Release studies

The release studies of polyphenols from freshly prepared hydrogel microbeads were performed at laboratory conditions. About 5 g of microbeads was suspended in 12,5 mL of distilled water. The samples were submitted to continuous agitation on an orbital shaker operating at 100 rpm (New Brunswick Scientific Co., Inc., Edison, NJ). At defined time intervals (2, 5, 10, 20, 30, 45, 60 min), an aliquot (70 µL) was taken for analysis of TPC.

RESULTS AND DISCUSSION

The obtained hydrogel alginate and alginate-inulin microbeads were analysed by optical microscope. Alginate microbeads encapsulating *P. tridentatum* extract appeared spherical with a quite smooth surface and average diameter of ~500 µm (Fig. 1a). Alginate microbeads with 10 mass% of inulin were slightly distorted from a perfect sphere and they are larger compared to alginate microbeads, ~700 µm (Fig. 1b). Alginate microbeads with 20 mass% of inulin had spherical appearance with the average diameter of ~800 µm (Fig. 1c).



(a) (b) (c)

Figure 1. Photos of alginate microbeads encapsulating *P. tridentatum* extract: (a) alginate microbeads; (b) alginate microbeads with 10 mass% of inulin; (c) alginate microbeads with 20 mass% of inulin.

After the freeze-drying, removed water accounts for 93,9, 86,0 and 78,6% of the initial weight for alginate, alginate with 10 mass% and 20 mass% of inulin, respectively. Microbeads treated by freeze-drying process were analysed by SEM. SEM was used to give information about features of the surface and the influence of inulin on surface morphology. Freeze-drying damaged the walls of alginate microbeads so they got irregular shape, with the surface of a spongy texture (Fig. 2 Ia, Ib, Ic). The problem of the gel collapse during freeze-drying process was significantly reduced by addition of inulin. The microbeads with 10 mass% (Fig. 2 IIa, IIb, IIc) and 20 mass% (Fig. 2 IIIa, IIIb, IIIc) of inulin have a rather smooth surface with a preserved round structure. SEM micrographs were also analysed using ImageJ application. The outcomes showed that the average diameter of alginate freeze-dried microbeads was 212,5±32,6 μm. Microbeads with 10 mass% of inulin, had average diameter 553,9±72,3μm and the one with 20 mass% of inulin, 578,8±83,5μm. The average diameters were determinate on a sample of 100 microbeads.

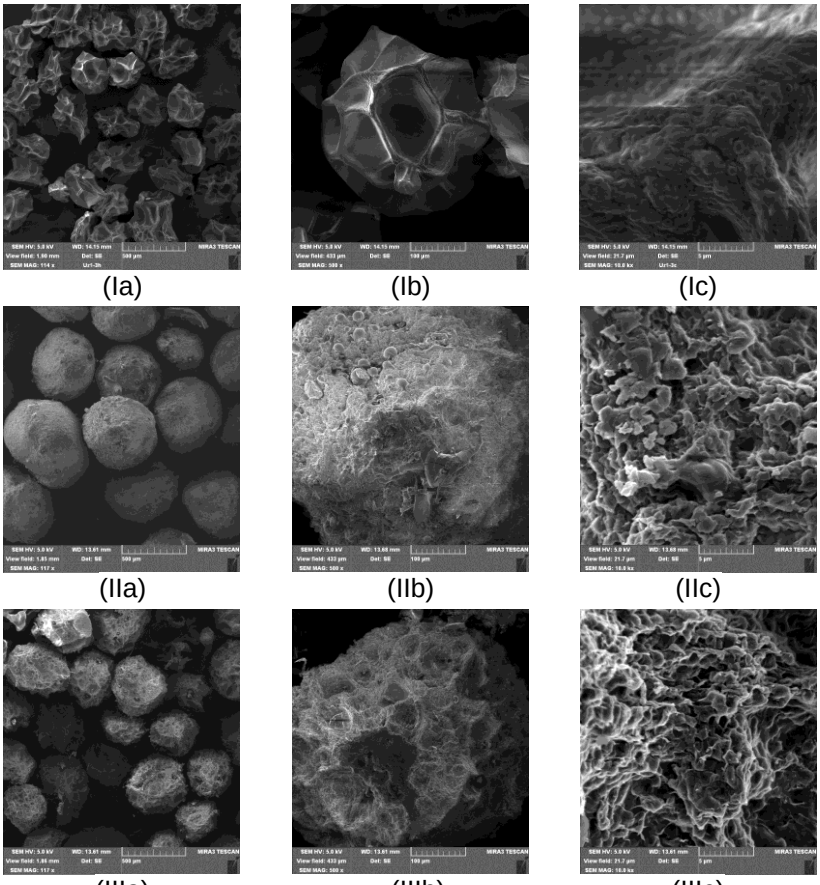


Figure 2. SEM micrographs of the freeze-dried microbeads: (I) alginate microbeads, (II) alginate microbeads with 10 mass% of inulin, (III) alginate microbeads with 20 mass% of inulin, (a) low magnitude, (b) medium magnitude, (c) high magnitude

The results of TPC and EE% for all microbeads are given in table 1. Depending on the addition on inulin, TPC in microbeads ranged from 0,24 to 0,33 mg GAE g_{beads}^{-1} and the highest amount of TPC was detected in alginate microbeads with 20 mass% of inulin. The results indicate that this type of microbeads has the highest encapsulation capacity. This can be explained by reduction of pore size of alginate in the presence of fillers (Rassis et al., 2002). In that way prevention of leakage of the encapsulated compounds was achieved.

Table 1. TPC, DPPH and ABTS of *P. tridentatum* extract and TPC, EE%, DPPH and ABTS of *P. tridentatum* extract encapsulated in hydrogel microbeads

SAMPLE	TPC (mg GAE g_{beads}^{-1})	EE(%)	DPPH(%)	ABTS(%)
Fresh extract	0,35[a]	/	57,5	47,7
Alginate microbeads	0,24	49,0	33,6	30,3
Alginate microbeads with 10 mass% of inulin	0,30	63,8	39,5	31,5
Alginate microbeads with 20 mass% of inulin	0,33	73,8	40,7	34,6

[a]*The unit is mg GAE mL^{-1}*

In this study, the antioxidant capacity of microbeads was determined by two analytical assays: (a) by ABTS radical cation ($ABTS^{*+}$) decolourization assay and (b) by DPPH radical photometric assay. The results are expressed as the percentage of radical inhibition and compared to antioxidant activity of the fresh extract (Tab. 1). The inhibition of the DPPH• radical with fresh extract (2 mg ml^{-1}) was around 57% and the inhibition of ABTS• was 47%. Upon encapsulation, antioxidative activity of extract compounds was preserved at a high level, as confirmed by both assays and alginate microbeads with 20 mass% of inulin showed the highest antioxidative potential.

FTIR was used to identify functional groups and characterise the relationship between the matrix and the extract components. In spectra of alginate freeze-dried microbeads (Fig.3) the strong and broad absorption band has been observed at 3430 cm^{-1} due to –OH stretching. This peak is slightly wider and moved to 3427 and 3417 cm^{-1} for samples with 10 and 20 mass% of inulin, respectively, due to overlap with the corresponding band in the case of inulin, located at 3380 cm^{-1} (pure inulin spectrum is not shown). The strong asymmetric stretching absorption band at 1625 cm^{-1} and weaker symmetric stretching band near 1440 cm^{-1} appeared due to the presence of carboxylate anions COO^- (Singh et al., 2009; Vijaya et al., 2008). The weak peak at 2920 cm^{-1} becomes stronger with increase in the amount of inulin, because this peak is related to the $–CH_2$ groups present in both, alginate and inulin. Similarly, the band at 1030 cm^{-1} becomes stronger and sharp, due to the C-O-C stretching of inulin (Fares et al, 2011). There are some changes in the peak intensity in the range of 900-1500 cm^{-1} between FTIR spectra of the native (not shown) and extract-encapsulating microbeads. The results of FTIR analyses indicate the absence of chemical interactions between extract compounds and alginate, thus it can be deduced that alginate hydrogel is a compatible material for encapsulating biochemical active compounds extracted from plants.

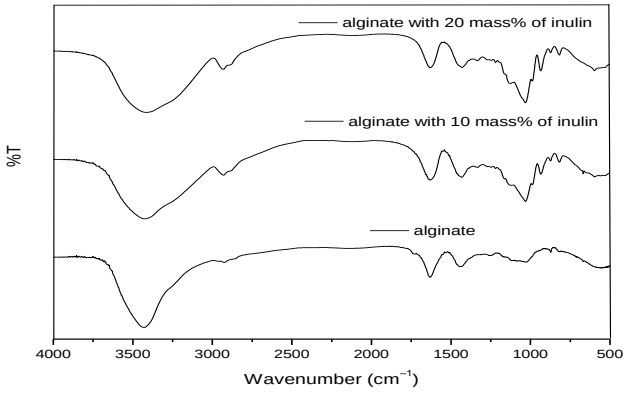


Figure 3. FTIR spectra of microbeads with encapsulated *P. tridentatum* extract

The release profiles for all three types of microbeads encapsulating *P. tridentatum* extract are shown on Fig. 4. The polyphenolic compounds were released after 30-40 min and the presence of inulin enabled better retention of polyphenols, especially in the first few minutes. For sake of comparison, two minutes after sinking microbeads in water, almost 90% of polyphenols was already released from alginate microbeads, while at the same time, about 30% was still captured within the alginate matrix containing 20 mass% of inulin.

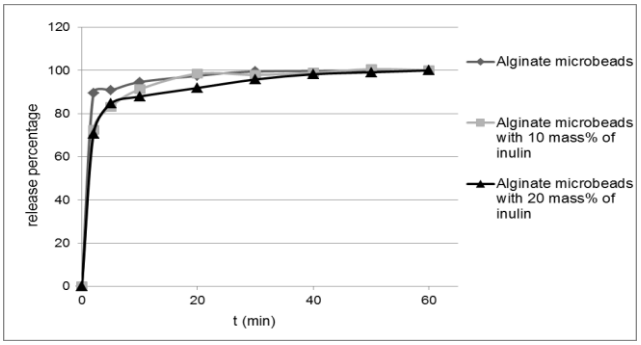


Figure 4. Release profiles of polyphenols from freshly prepared microbeads encapsulating *P. tridentatum* extract.

CONCLUSIONS

Encapsulation of aqueous *P. tridentatum* extract within alginate and alginate-inulin microbeads has been assessed. The obtained microbeads displayed significant polyphenol content. The best results were achieved with alginate-inulin microbeads, as they were treatable by freeze-drying. They were also richer in polyphenols and they protected the extract from release better than plain alginate forms. The antioxidant activity of the extract was preserved after microencapsulation at a high level, especially in case of alginate microbeads with 20 mass% of inulin.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education and Science, Republic of Serbia (Project No. III46010 and Serbia–Portugal bilateral agreement 2011-2012).

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QUANTIFICATION OF VIABLE SPRAY-DRIED *LACTOBACILLUS PLANTARUM* TA AND 7A AFTER TWO YEARS OF STORAGE BY USING REAL-TIME PCR

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ABSTRACT: Probiotic are "living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition", and it is recommended that probiotic products contain at least 10^7 live microorganisms per g or per ml. Spray-drying is one of the possibilities to improve the survival of probiotic bacteria against negative environmental effects. The aim of this study was to compare survival of two spray-dried *Lactobacillus plantarum* TA and *Lactobacillus plantarum* 7A strains isolated from plant and breast-fed baby's feces. In addition, beside the plate-count technique, the aim was to examine the possibility of using propidium monoazide (PMA) in combination with real-time polymerase chain reaction (real-time PCR) for determination of spray-dried *Lb. plantarum* strains after two years of storage at 4°C. Both tested strains showed very good ability to survive spray-drying. After two years of storage at 4°C, the number of intact cells, *Lb. plantarum* TA and *Lb. plantarum* 7A, was determined by real-time PCR with PMA, and it was similar to the number of investigated strains obtained by plate count method. The spray-drying was effective in maintaining the viability of *Lb. plantarum* TA and 7A strains after two years of storage at 4°C. The PMA real-time PCR determination of the viability tested strains could complement the plate count method.
Key words: *real-time PCR, propidium monoazide, probiotic bacteria*

INTRODUCTION

During the last few decades, a wide variety of novel products containing probiotics have been developed. A number of definitions of the term "probiotic" have been used over the years, but the one derived by the Food and Agriculture Organization (FAO/WHO, 2002) best exemplifies the breadth and scope of probiotics as they are known today: "Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" (Reid, 2006). They are able to survive the low pH of the stomach, which is normally destructive for most microbes (Holzapfel et al., 1998) and to contribute positively in the activity of intestinal microflora. The most probiotic bacteria belong to the *Lactobacillus* genera and *Bifidobacterium* strains (Prasad et al., 1998).

In the recent past, there has been an explosion of probiotic health-based products. Many reports indicated that there is poor survival of probiotic bacteria in these products (Shah, 2000; Lourens-Hattingh and Viljoen, 2001). Several approaches have been adopted in endeavoring to improve probiotic survival in traditional probiotic foods. Freeze-drying and spray-drying are most commonly used microencapsulation methods. The process of spray-drying is economical, can be operated on a continuous basis, it is easily scaled up and uses equipment readily available in the food industry (Gibbs et al., 1999). The disadvantage is that the high temperature used in the process of spray-drying, may not be suitable for encapsulating probiotic bacterial cultures. However, proper adjustment of the processing conditions, such as the inlet temperature, can achieve viable encapsulated cultures.

Viability is generally considered a prerequisite for optimal probiotic functionality (Maukonen et al., 2006). Many studies have shown that the viability of bacteria is not a simple question of cells being dead or alive (Nystrom, 2001; Bogovic Matijasic and Rogelj, 2006). Plate counting is one of the classic and most widespread techniques for determining the viability of

spray-dried bacteria, though there are obvious disadvantages, such as limiting microbial recovery and sensitivity and relatively long times needed for the growth of colonies. Therefore, during the recent years there has been increasing interest in the development of culture-independent molecular methods for identification and quantification of probiotic bacteria. Quantitative real time polymerase chain reaction (real-time PCR) is the most widely applied technique for direct quantification of bacteria in mixed samples (Juste` et al., 2008). However, at the moment, there are still some limitations to overcome before the introduction of such methods in routine analysis. Real-time does not enable the distinction between DNA arising from dead or alive cells; therefore, the DNA from dead cells contributes to the results. A possible way to distinguish between alive and dead cells is the integrity of membrane. Viable cells with intact membranes are impermeable to the passage of certain intercalating DNA agents, which, however, selectively penetrate dead cells. Propidium monoazide (PMA) has been shown to be useful for differentiating between some live-dead Gram-positive and Gram-negative bacteria (Nocker et al., 2006). PMA is a DNA-intercalating dye with the azide group, which enables covalent binding to DNA under bright visible light and, consequently, strongly inhibits PCR amplification.
The aim of this study was to compare survival of two spray-dried *Lactobacillus plantarum* TA and *Lactobacillus plantarum* 7A strains isolated from plant and breast-fed baby's feces. Furthermore, beside the plate-count technique, aim was to examine the possibility of using PMA in combination with real-time PCR with SYBR Green I chemistry for selective quantification of live spray-dried tested strains after two years of storage at 4°C.

MATERIAL AND METHODS

Strains and culture conditions

Strains *Lb. plantarum* TA and *Lb. plantarum* 7A isolated from plant and breast-fed baby's feces, belong to the strain collection of the Department for Industrial Microbiology, Faculty of Agriculture, University of Belgrade, Serbia. The strains were cultured in MRS broth (Merck, Darmstadt, Germany) at 37ºC. For both strains, cell enumerations were carried out on MRS agar (Merck, Darmstadt, Germany) 48 h at 37˚C under anaerobic conditions (Gas Pak, BBL, Germany).

Spray-drying

Spray-drying tests were performed using the method of Petrovic (2011). Overnight cultures (300 ml) were centrifuged (4500 x g, 15 min, 15˚C). The pellet was washed twice in 50mM K2HPO4 (pH 6.5) and resuspended in 300 ml of sterile RSM (20% w/v). Both tested strains were spray-dried with a laboratory scale spray-dryer (model B-290 Buchi mini spray dryer, Switzerland) by using the constant inlet air temperature of 170˚C and the outlet temperature of 80˚C.

Determination of viability of tested strains in spray-dried powders

Plate count method

The colony forming units (CFU) of cultures were assessed by the plate count using MRS agar. 9 ml of Na-citrate (2% w/v) was added to 1 g spray-dried powder and the preparation allowed to rehydrate before further dilutions were performed and appropriate dilutions plated (1ml) onto MRS agar. The plates were placed in anaerobic conditions and incubated at 37˚C for 48 h (Gas Pak, BBL, Germany). The number of bacteria before drying was determined and compared with the number of bacteria per spray-dried powders, CFU/g dry weight was determined from CFU/ml after drying a known volume of the original sample used for plating.

Real-time PCR for selective quantification

Sample preparation

Both spray-dried tested strains were well resuspended in Na-citrate (2%) solution to prepare 1% (w/w) solution. One milliliter of 1% spray-dried suspension was centrifuged (5000 x g, 10

min), and DNA was extracted from pellet by the method described below. Samples were tenfold serially diluted prior to PCR analysis.

PMA treatment

Spray-dried tested lactobacilli were treated with PMA, as described by Nocker et al. (2006). PMA (Biotium, Inc, CA, USA) was dissolved in dH2O to create 20 mM PMA stock solution and stored at -20˚C in the dark. Powders of each tested strain were resuspended in Na-citrate solution to prepare 1% (w/v) solution. An adequate volume of PMA stock solution was added to 500 µl of aliquots of spray-dried cultures to make the final concentration of 50 µM. This was followed by an incubation period of 5 min of 5 min in the dark with occasional mixing to allow the PMA to penetrate the dead cells and to bind to the DNA.

Samples were light-exposed to a halogen light source (650W, 150V) for 5 min and samples were laid horizontally on ice to avoid excessive heating and placed about 20 cm from the light source. After photo-induced cross-linking, the cells were pelleted at 5000 x g for 10 min prior to DNA isolation by the bellow described protocol.

DNA isolation

Total genomic DNA from the samples was extracted using MaxwellTM 16 cell tissue DNA purification kit applied with MaxwellTM 16 instrument (Kramer et al., 2009). 400 µl of TE buffer and 100 µl of lyzozyme (25 mg/ml) with mutanolysine (10 U/ml) were added to the pellet, which was further resuspended and incubated for 2 h at 37˚C for bacterial cell lyses, before isolation by the Maxwell system. The whole volume of prepared samples was transferred into the first well of the cartridge and further treated according to the manufacturer`s instructions. Finally, DNA was resuspended in 300 µl of elution buffer with added 1.5 µl RNAse (4mg/ml).

Real-time PCR

PCR amplifications were performed with an MX3000P (Strategene, LA Jolla, CA, USA) instrument (Kramer et al., 2009). The reaction mixture (25 µl) containing Platinum SYBR Green qPCR Super Mix UDG (11733; Invitrogene, Carlsbad, CA, USA), 0.2 µM of each primer and 5 µl of genomic DNA diluted -10 fold. The following primers were used LactoR`F (5`-CACAATGGACG(A/C)AAGTCTGATG-3`) and LBFR (5`-CGCCACTGGTGTTCTTCCAT-3`) (Songjinda et al., 2007). The amplification programme was 50˚C for 2 min and 95˚C for 2 min, 35 cycles of 95˚C for 30 s, 60˚C for 15 s, 72˚C for 20 s, and then 95˚C for 1 min and 55˚ for 30 s.

Statistical analysis

The Ct values were automatically generated by the Invitrogene software. Experiments were replicated at least three times. Means and standard deviations were calculated using one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Viability of tested strains in spray-dried powders determined by plate counting

The main factor that governs microbial survival during spray-drying is the ability of the strain to survive high temperatures. The heat resistance experiments demonstrated that stationary-phase cultures are more resistant to heat stress than cells in exponential growth phase (Texeira et al., 1994). In relation to that it should be mentioned that both tested strains were in stationary phase.

The viability of strains, *Lb. plantarum* TA isolated from plant and *Lb. plantarum* 7A isolated from breast-fed baby's feces, in spray-dried powders are shown in Table 1. The survival rates of tested *Lb. plantarum* TA and *Lb. plantarum* 7A strains after spray-drying, determined by plate counting, were very high, 91.00% and 85.20%, respectively. *Lb. plantarum* TA decreased slightly more than 1.0 log, while *Lb. plantarum* 7A decreased less than 2.0 log after spray-drying.

Table 1. Viability of tested strains in spray-dried powders

Strains	Before spray-drying log (CFU/g)[a]	After spray-drying log (CFU/g)[a]	Percent of survival (%)
Lb. plantarum TA	11.23 ± 0.07	10.20 ± 0.12	91.00%
Lb. plantarum 7A	12.61 ± 0.04	10.75 ± 0.03	85.20%

[a] *Mean values (log CFU/g) and standard deviation were calculated from three parallel plate count analyses*

Viability of tested strains in spray dried powders after two years of storage at 4ºC

Different methods were used for the enumeration of probiotics in the products containing dried probiotic bacteria. As an alternative for the most common plate count methods, real-time PCR methods have been used for the quantification of probiotic bacteria in several studies, however, most often in different foods, such as fermented milk products, while studies reporting the application in probiotic food supplements are few (Grattepanche et al., 2005). In some studies, the real-time PCR method was successfully used for the enumeration of LAB in lyophilized dairy starter cultures (Friedrich and Lenke, 2006).

One of the aims of this study was to evaluate the possibility of using PMA in combination with real-time PCR using SYBR Green I chemistry for the selection of viable lactobacilli in spray-dried powders. Optimal conditions of PMA treatment for enumeration of viable cells of tested strains were 50µM PMA, 5 min incubation, 5 min photoactivation (Fujimoto et al., 2010; Noecker et al., 2007). Bacterial counts derived from real-time PCR determination of PMA-treated and non-treated samples of spray-dried tested strains were compared to the plate count method (Table 2).

Table 2. The number of cells of spray-dried tested strains performed by the plate count method, real-time polymerase chain reaction (PCR), and propidium monoazide (PMA) real-time PCR after two years of storage at 4°C

Strains	Plate count[a]	Real-time PCR without PMA[b]	Real-time PCR with PMA[b]
Lb. plantarum TA	9.80 ± 0.04	9.68 ± 0.41	9.34 ± 0.42
Lb. plantarum 7A	9.49 ± 0.14	9.75 ± 0.08	9.13 ± 0.42

[a] *Mean values (log CFU/g) and standard deviation were calculated from three parallel plate count analyses*

[b] *Mean values (log CFU/g) and standard deviation calculated from Ct values; based on two parallel DNA extracts from which two real-time cycles were run*

Spray-dried *Lb.plantarum* TA strain showed reduction in cell counts less than 2 log, while spray-dried *Lb. plantarum* 7A strain showed reduction in cell counts higher than 3 log, after two years of storage at 4°C, determined by plate count method. The concentrations log (CFU/g) of tested strains obtained by plate count method were not significantly ($p < 0.05$) different from the values obtained by real-time PCR analysis. The comparison of plate count real-time PCR for enumeration of LAB commercial milk products showed that the loss of cultivability during storage was train dependent (Furet et al., 2004). For instance, when it comes to the *Lactobacillus casei* group, the results of real-time PCR quantification did not differ from the results of the plate count, while for most of the strains of *Lb. delbrueckii*, ten to 100 lower values were detected by plate counting (Kramer et al., 2010). The treatment of samples with PMA, followed by real-time PCR analysis, as presented in this study, appears a promising approach for a routine method of enumeration of probiotic bacteria in spray-dried powders.

CONCLUSION

Both tested strains have shown very good ability to survive process of spray-drying. Further, the results of this study indicated that the spray-drying process of microencapsulation offered protection to the tested strains during storage for two years at 4°C. Because of the

complexity of the bacterial population, including physiological states of bacteria, the combination of different methods would represent a significant improvement of the analysis of bacteria. The real-time PCR approach will be further improved in the way that will be enable to distinguish between DNA derived from intact or damaged cells.

ACKNOWLEDGEMENTS

The authors of this paper highly appreciate the financial support from the Ministry of Education and Science of the Republic of Serbia (National Project No. 046010 (2011-2014) and Bilateral Project, Republic of Serbia and Republic of Slovenia (2010-2011), BI-SI/10-11-035).

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ENCAPSULATION OF BASIL (*OCIMUM BASILICUM*) ESSENTIAL OIL

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ABSTRACT: Basil (*Ocimum basilicum*) is an aromatic plant widely used as a culinary herb, commonly added as fresh. There are many scientific studies that have confirmed health benefits of the basil oil. Bioactive compounds of the essential oils found in basil have antioxidant and antimicrobial properties. However, effectiveness of these compounds depends on preserving their stability, which can be increased by encapsulation.
The aim of this study was to encapsulate the basil essential oil (EO) in alginate microbeads in order to protect and stabilize bioactive compounds in it. Calcium alginate microbeads entrapping the EO were produced by electrostatic extrusion technique. The obtained microbeads were characterized from the aspect of total phenol content, encapsulation efficiency and antioxidant capacity. Total polyphenol content (TPC) of microbeads was analyzed by the Folin-Ciocalteu reagent. Encapsulation efficiency was calculated as the ratio between the TPC in the citrate solution of dissolved microbeads and the TPC of the initial EO. The radical scavenging activity was determined as Trolox equivalent antioxidant capacity (TEAC) and by using stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The rehydration properties of air dried calcium alginate gel microbeads were investigated in water and phosphate buffer solution. Also, samples were analyzed by optical microscopy (OM).
Encapsulation efficiency was obtained to be approximately 60 %. The results of TEAC and DPPH tests indicate that antioxidant activity was preserved at a satisfactory level. The average diameters of fresh and dried microbeads were 860.8±44.9 and 416.0±37.2 μm, respectively. The particles, with and without EO, rehydrated in buffer were highly swollen, from 5000 to 10000 %w/w.
The results suggest that alginate microbeads encapsulating EO appeared to be suitable dosage forms. Thus, possible applications of these microbeads could be in the production of functional foods.
Key words: *basil, essential oil, encapsulation, alginate microbeads, rehydration*

INTRODUCTION

Basil (*Ocimum basilicum*) is a popular culinary herb in many traditional cuisines and diets. Also, this plant is a source of valuable essential oil and extracts used in cosmetic industry and pharmacy. Basil is well known as a source of antioxidant, antimicrobial and antitumor active compounds. The activity of basil essential oil and extracts is mainly connected to a presence of phenolic and aromatic compounds (Mäkinen & Pääkkönen, 1999; Holm, 1999; Taie et al., 2010). During production and storage, some of the active compounds can be permanently damaged, since most of these compounds are too sensitive to many factors. The effectiveness of these valuable active compounds can be protected using encapsulation processes. Encapsulation is defined as a process of entrapping active compounds within another substance (Zuidam & Shimoni, 2010). Thus, encapsulation is an effective method to protect bioactive compounds, preserve their stability, and prevent some interactions with food matrix which are undesirable (Nedović et al., 2011).
Electrostatic extrusion is a promising encapsulation technique for production of spherical beads loading active compound. This technique has been intensively used for encapsulation of cells, plant extracts as well as food additives in calcium alginate matrix (Nedovic et al.,

2001; Manojlović et al., 2008; Stojanović et al., 2011; Belščak-Cvitanović et al., 2011). The objective of this study was to investigate the encapsulation of basil EO in calcium alginate matrix using electrostatic extrusion. The efficiency of encapsulation procedure was evaluated by comparison of both, total polyphenols content and antioxidant activity of free and encapsulated basil EO. Also, the dehydration and rehydration properties of the samples were analyzed and microbeads were observed under optical microscope.

MATERIALS AND METHODS

In this study, sodium-alginate (medium viscosity) purchased from Sigma was used for a matrix preparation. Calcium chloride dihydrate was purchased from Analytika (Czech Republic). Folin-Ciocalteu, Na-carbonate, and Na-citrate were of analytical grade and supplied by Sigma-Aldrich (St. Louis, MO, USA). 2,2′-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid)diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium chloride, potassium persulfate, monosodium phosphate monohydrate and disodium phosphate heptahydrate were obtained from Sigma-Aldrich (Germany).

Plant material and cultivation conditions

The seeds of *O. basilicum* subs. *basilicum* var. *glabratum* cultivar Cinnamon were planted in the greenhouse in March of 2009, and stuffed with commercial substrate (Beatović et al., 2006). Seedling production lasted 48 days. During May of 2009, nursery plants were transplanted into the experimental field in a randomized complete block design with four replicates. The harvest of plants was performed at the full flowering stage in June of 2010. Plants were air dried in the shade, and then packed and stored. Seeds of *O. basilicum* subs. *basilicum* var. *glabratum* cultivar Cinnamon were obtained from the Institute for Crop Sciences of the Faculty of Agriculture in Belgrade and the Plant Gene Bank of Serbia where plants were designated and deposited under DB codes.

Extraction of basil essential oil

The extraction of the EO was carried out in order to extract and quantify the amount of EO present in dried basil herbs. A weight of 20 g of dried herbs was mixed with 400 mL of distilled water and subjected to hydro-distillation for 2,5 hours using a Clevenger apparatus. The volume of the extracted EO was determined and recorded. The extracted oil was dehydrated over anhydrous sodium sulfate and stored in a glass vials at 4°C.

Determination of total phenol content (TPC)

Total phenolic content was determined spectrophotometrically using the Folin-Ciocalteau reagent, according to the method of Singleton et al (1998). In brief, 125 µL of the sample was pipetted into a volumetric flask containing 125 µL of Folin–Ciocalteu reagent, 0,5 mL of distilled water and after 6 minutes 1,25 mL of 7% (w/v) Na-carbonate was added, and the volume was made up with 3 mL of distilled water. After 90 minutes, the absorbance of the blue colouration was measured at 760 nm against a blank sample. Gallic acid was used as the standard and the results expressed as mg L^{-1} of gallic acid equivalents (GAE).

Determination of free radical-scavenging ability (DPPH)

The DPPH radical scavenging activity of the basil EO was determined according to Molyneux (2004). The DPPH• solution in ethanol ($5x10^{-5}$M) was mixed with 100 µL sample, incubated for 30 minutes in dark and then the decrease in absorbance at 517 nm was measured (A_s). A blank sample contained 100 µL of ethanol in the 3mL of DPPH• solution (A_B). Radical scavenging activity was calculated using the following formula:

$$\% \text{ Inhibition} = ((A_B - A_s)/A_B) \times 100$$

where (A_B) is absorbance of the blank sample and (A_s) is absorbance of the sample.

Trolox equivalent antioxidant capacity (TEAC)
The free radical scavenging capacity of the EO was also studied using the ABTS radical assay (Re et al. 1999). ABTS• was produced by mixing 5 mL of ABTS water stock solution (7 mM) with 88 µL of potassium persulfate (140 mM) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS• solution was diluted with ethanol to an absorbance of 0.7 (±0.02) at 734 nm. An appropriate solvent blank reading was taken (A_B). After the addition 30 µL of the EO solutions to 3 mL of ABTS• solution, the absorbance reading was taken 6 minutes after initial mixing (A_s).

Encapsulation of basil essential oil
Encapsulation of basil essential oil in calcium alginate matrix using electrostatic extrusion was realized according to procedure described by Nedović et al. (2001), with some modifications. The first step in encapsulation process was a preparation of a shell solution by dissolving sodium alginate powder in distilled water (0.02g/mL). Alginate/EO emulsion (2.5% w/w of EO) was prepared using Ultra-Turrax T25 (T25 digital ULTRA-TURRAX®, IKA, Germany) at a speed of 10000 rpm for 5 minutes. Emulsion was extruded through blunt stainless still needle (22 gauge) using a syringe pump (Razel, Scientific Instruments, Stamford, CT) under constant flow rate of 70mL/h. Electrostatic potential (6.0kV) was formed by high voltage dc unit (Model 30R, Bertan Associates, Inc., New York). The collecting solution was calcium chloride (0.015 g/mL). The distance between the needle tip and the collecting solution was 2.5 cm. After extrusion, the beads were left in the collecting solution for 45 minutes. After gelling period, microbeads were rinsed with distilled water. In order to produce dried encapsulated samples loading essential oil, wet beads were air-dried at room temperature to a constant mass.

Encapsulation efficiency (EE)
Encapsulation efficiency (EE) was calculated as the amount of TPC encapsulated in microbeads (m_b) divided by the TPC of the solution used for the preparation of microbeads (m_s), according to the formula:

$$EE\% = m_b / m_s \times 100$$

Quantification of TPC in microbeads (m_b) was performed using dissolved microbeads in 2% (w/v) sodium citrate solution, in a weight ratio of 1:4, using a Vortex mixer (Tehnica zeleznik EV-102) to chemically dissolve them at room temperature. TPC was determinated using the Folin–Ciocalteu method.

Bead size measuring
The diameter of beads was determined microscopically using binocular microscope DMLS Leica (Leica, Germany) equipped with a camera DC 300 (Leica, Germany) and a software for measuring IM 1000 (Leica, Germany). At least 50 microbeads were measured and the average diameter and standard deviation were calculated.

Drying kinetics
Drying kinetics study was carried out by measuring weight loss of alginate microbeads during the time. The wet microbeads were air-dried at room temperature and weight loss of the samples was measured at defined intervals of time on an analytical balance until the constant mass was achieved.

Rehydration of dried microbeads
The same amounts of air dried microbeads with and without basil EO were placed in a (1) 20 mL of distilled water and in a (2) 20 mL of phosphate buffer (pH=7.4), and then shaken at a speed of 100 rpm. Samples were measured during 420 minutes at definite intervals of time.

RESULTS AND DISCUSSION

Extraction of basil essential oil

Essential oil extracted from basil dried herbs was light yellow coloured with the specific aroma. The total content of essential oil in dried herbs was 1.0%. Generally, oil yield for basil is in the range 0.2-1.0%, which depends on plant physiology and source. According to the results, basil plantation for production of essential oil can be effective in ecological conditions of Serbia.

Encapsulation efficiency

The results of TPC, DPPH, TEAC of basil EO and TPC, EE%, DPPH, TEAC of alginate microbeads loaded with basil EO are given in Table 1.

Table 1. TPC, DPPH and TEAC of basil EO and TPC, EE%, DPPH and TEAC of alginate microbeads loaded with basil EO

test / sample	Basil EO	Alginate microbeads loaded with basil EO
TPC(mgGAE/gDW)	7.54	4.53
TEAC(%)	67.40	39.30
DPPH(%)	74.50	48.50
EE(%)	-	60.13

According to the results, the content of polyphenols as well as antioxidant capacity (expressed in both terms, DPPH and TEAC) of encapsulated basil EO were proportional to the encapsulation efficiency.

Bead size measuring

The average diameter of freshly-prepared beads encapsulating basil essential oil was 860.8±44.9 μm (Fig 1-a). After drying, the contraction of beads occurred, probably as a result of water evaporation (Fig 1-b). The mean diameter of dried beads (416.0±37.2 μm) decreased for about 50% compared to freshly-prepared beads. Also, the changes in beads shape during drying process were noticed, such as distortion of sphericity. According to the literature, the distortion of sphericity and gel collapse during drying could be prevented by addition of filler substances (Zohar-Perez et al., 2004).

Figure 1. Photos of beads loading basil essential oil: (a) wet calcium alginate beads with encapsulated EO; (b) dried calcium alginate beads with encapsulated EO

Drying kinetics

Drying is a way of food preserving frequently used in the food industry. However, drying and subsequent rehydration often results in a decline of food quality. Therefore, in this study we examined the rates of drying and rehydration of alginate microbeads encapsulating basil essential oil and compared to those of plain alginate microbeads. Fig 2. shows weight loss of alginate microbeads with and without basil essential oil during 30 hours of drying. Constant weights were achieved after 8 hours.

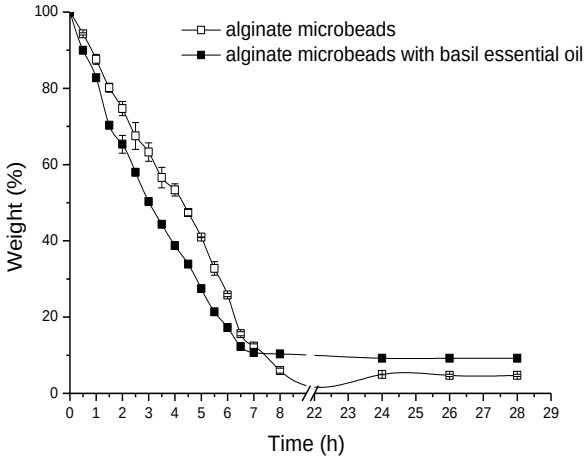

Figure 2. Weight loss of alginate microbeads and alginate microbeads with basil EO during drying process.

Rehydration of dried microbeads

As shown in Fig.3-A equilibrium was reached after 3 - 4 hours and the particles rehydrated in phosphate buffer were highly swollen. For example, after 90 minutes particles swelled about 60 times. However, after approximately 4 hours unfilled particles continued to swell more than 10000 %w/w, while particles with EO after same period started to decompose. In general, rehydration of calcium alginate beads is dependent on the ionic strength of the rehydration medium as well as on alginate source (i.e. mannuronic to guluronic acid ratio). At the high salt concentration such as one of the phosphate buffer used in this study (100 mM and pH=7.4) disintegration of alginate beads occurs after several hours of hydration; this happened faster in case of alginate microbeads encapsulating basil oil. According to our results (Fig.3-B) and literature data (Vreeker et al, 2008), calcium alginate beads do not rehydrate to a significant extent in pure water (i.e. in the absence of salt).

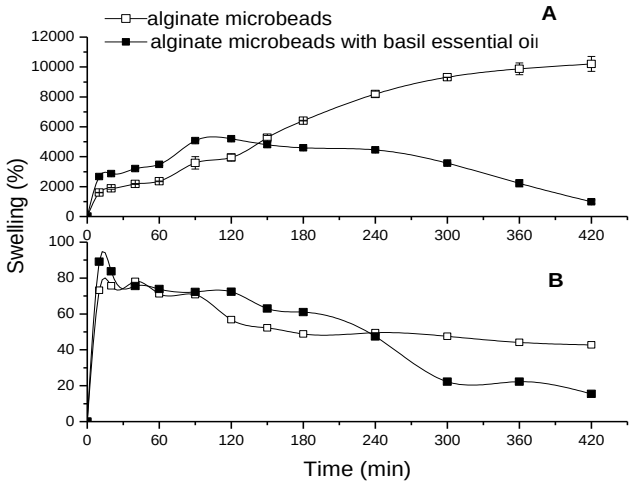

Figure 3. Swelling of alginate microbeads and alginate microbeads with basil EO (A) in phosphate buffer and (B) in water

CONCLUSIONS

Basil is widely used in the culinary and the food processes as an aroma to improve the taste, odor, and digestability of food products. The results show that basil EO has a signficant antioxidative potential and total polyphenol content. According to the results, the antioxidant activity of the basil EO was preserved after encapsulation at a high level. As shown,

encapsulation of the basil EO in alginate microbeads is suitable for protection and controlled delivery of functional compounds.

ACKNOWLEDGMENTS

This research was supported by the Ministry of Education and Science, Republic of Serbia, Project No. III 46001.

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STILLAGE FROM BIOETHANOL PRODUCTION AS SUBSTRATE FOR PARALLEL PRODUCTION OF LACTIC ACID AND BIOMASS

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ABSTRACT: Stillage as a by-product of bioethanol production on starch substrates is rich in proteins and minerals essential for lactic acid bacteria (LAB) growth. Parallel production of lactic acid and biomass for animal feed on the stillage could bring additional value to the bioethanol production process and resolve environmental problem of the stillage disposal. Fermentations were preformed on liquid and whole stillage from bioethanol production on wasted bread with a strain *Lactobacillus rhamnosus* ATCC 7469. The stillage was obtained from ethanol factory Reahem, Srbobran and sterilized (121°C/103kPa/15 min). After adjustment of reducing sugar concentration with sterile 70% (w/w) glucose solution, the media was inoculated with 5% (v/v) of the overnight *L. rhamnosus* ATCC 7469 culture. Effect of different initial sugar concentrations was studied in batch fermentations at 41 ^{o}C, under microaerophilic conditions, with shaking (90 rpm). Lactic acid concentration (enzymatic method), reducing sugar concentration (spectrophotometric method according to Miller) and a number of viable cells (pour plate technique) were determined during the fermentation. Under selected conditions the highest yield of lactic acid of 92.7% was achieved on whole stillage with initial sugar concentration of 55 g/l. The number of viable cells at the end of fermentation was above 10^9 CFU/ml. Whole stillage from bioethanol production could be used without nitrogen or mineral supplementation as a substrate for parallel production of lactic acid and biomass. Spent fermentation media enriched with LAB biomass may be used as a valuable animal feed.
Keywords: *lactic acid, fermentation, Lactobacillus rhamnosus ATCC 7469, stillage, probiotic biomass, animal feed*

INTRODUCTION

Fermentative production of chemicals for food industry on cheap substrates is important for sustainable food manufacturing. Lactic acid is used in food industry as an acidifying agent, flavour and preservative due to its antimicrobial activity. Polymers of lactic acid (PLA) are extensively utilized for biomedical purposes (implants, suture materials, bone fixations, tissue engineering) (Bastiol, 1997) and as a biodegradable packaging materials (Data and Henry, 2006). Today, fermentation by lactic acid bacteria is a main production route in industrial facilities. Starch feedstocks are extensively studied as substrates for lactic acid fermentations (Anuradha et al., 1999; Rojan et al., 2005); however, establishment of efficient processes on wastes and by-products is desirable, although challenging approach.
Stillage from bioethanol production is a complex waste product which has been previously used in fermentative processes for single cell protein, biogas and fertilizers production (Wilkie et al., 2000). In Serbia, bioethanol production is mainly based on starch substrates and molasses. Main by-product of bioethanol production is distillery stillage which had a significant negative environmental impact. Because of high BOD_5 values of 15-340 g L^{-1}, depending on a feedstock, and pour odour of stillage, it cannot be disposed without previous treatment (Pejin et al., 2009). Costs of its treatment significantly influence financial aspects of bioethanol production and stillage utilization could be a possible solution. Nutritional requirements of lactic acid bacteria employed in lactic acid production are fastidious, especially for nitrogen sources. Almost 38 % of total costs of lactic acid production are taken by costs of yeast extract, as a nitrogen source (Teleyadi and Cheryan, 1995). Stillage

remained after bioethanol production contains residuals of yeast cells from alcoholic fermentation rich in B vitamins and nitrogen and they have a proven positive effect on growth and productivity of lactic acid bacteria (Altaf et al., 2007). Lactic acid bacteria are also well-known as species with potentially probiotic characteristics (Naidu et al., 1999). The strain *Lactobacillus rhamnosus* ATCC 7469 was used for lactic acid fermentation of stillage in our study. This strain had shown *in vitro* adherence capability (Tuomola and Salminen, 1998) and it is recommended as safe for use in animal nutrition (EFSA, 2007). Intended use of spent fermentation media rich in *L. rhamnosus* biomass could bring significant additional value to the process.
In this study, the possibility of parallel lactic acid and animal feed production was investigated. Initial sugar concentrations were varied in order to obtain efficient lactic acid production and high concentration of viable *Lactobacillus rhamnosus* ATCC 7269 cells. The spent fermentation media with *L. rhamnosus* biomass media was subjected to low pH in order to evaluate the survival of this potentially probiotic biomass.

MATERIAL AND METHODS

Microorganism

Lactobacillus rhamnosus ATCC 7469 used in this experiment was obtained from American Type Culture Collection (ATCC, Rockville, USA). Stock cultures of lactic acid bacteria (LAB) were stored at -20 °C in 3 ml vials containing Man Rogosa Sharpe medium (MRS) (Fluka, USA) and 50 % (v/v) of glycerol as a cryoprotective agent. The culture was propagated under anaerobic conditions using Anaerocult ® C bags (Merck KGaA, Darmstadt, Germany) at 37 °C for 18 h in MRS broth before inoculation to fermentation medium. The fermentation was initiated by addition of 5 % (v/v) of the inoculum.

Lactic acid fermentation

A whole stillage (without separation of solid fraction) remained after bioethanol production on wasted bread was obtained from Reahem Ethanol Plant (Reahem, Srbobran, Serbia). The pH of the stillage was adjusted to 6.5 with 30 % solution of NaOH (Sigma-Aldrich, USA), and then sterilized (120 °C/15 min). Sugar concentration in the stillage was relatively low (12.6 g L^{-1}) and initial sugar concentrations were set at 55 g L^{-1}, 75 g L^{-1} and 85 g L^{-1} by addition of sterile 70 % glucose solution. The effect of different initial sugar concentrations on lactic acid and biomass production in batch fermentations was studied. All lactic acid fermentations were performed with shaking (150 rpm, KS 4000i control, IKA®, Werke GmbH & Co. KG, Staufen, Germany) at temperature of 41 °C. During the fermentation, pH was adjusted to 6.5 in all samples, and maintained at 6.5 by addition of 30 % solution of NaOH in four hour intervals. The fermentations were preformed in 500 ml flasks with 250 ml of the fermentation media under anaerobic conditions in the gas pack system. During the fermentation: pH, sugar consumption, lactic acid concentration and a number of living cells were analyzed.

Survival of *Lactobacillus rhamnosus* ATCC 7469 under the low pH

Survival of *L. rhamnosus* under the pH of 2.5 during three hours was studied in 100 ml flasks. Volume of 50 ml spent fermentation media with *L. rhamnosus* cells was transferred in the flask and pH was adjusted to 2.5 by addition of 1 M HCl. In a control sample (50 ml) pH was adjusted to 6.5 by addition of 30 % NaOH. Samples were taken in an hour long intervals and the number of viable cells was counted using pour plate technique on MRS agar after incubation for 48 h at 37 °C.

Analytical methods

The dry matter percent was determined by a standard drying method in an oven at 105 °C to constant mass (A.O.A.C., 2000). The protein content was estimated by Kjeldahl method as the total nitrogen and multiplied by factor 6.25 (A.O.A.C., 2000). The lipid content was determined by Soxhlet method and ash content was determined by slow combustion method

at 650 °C for 2 h (A.O.A.C., 2000). The concentration of reducing sugars, calculated as glucose, was estimated by 3, 5-dinitrosalicylic acid method using spectrophotometer, Ultraspec 3300 pro, Biochrom LTD, UK (Miller, 1959). Lactic acid concentration was determined by enzymatic method (L-/ D-Lactic acid assay, Megazyme®, Wicklow, Ireland) according to procedure prescribed in assay. Number of viable *L. rhamnosus* ATCC 7469 cells was estimated using pour plate technique. The chemicals used in experiments were analytical grade.

All experiments were done in triplicates. The values are expressed as means ± standard deviation. Mean values of treatments were compared by the analysis of variance (One-Way ANOVA) followed by Tukey test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The chemical composition of the stillage used in our study is presented in Table 1. Low concentration of sugars in the stillage is a result of previous complete alcoholic fermentation. The stillage was supplemented with glucose as a carbon source and initial glucose concentration was found as an important parameter for lactic acid and biomass production kinetics in following experiments. Relatively high amount of proteins (more than 50 % of dry matter) indicated that the stillage could be suitable as a substrate for growth of nutritionally fastidious microorganisms like lactic acid bacteria (LAB).

Table 1. Chemical composition of the stillage

Chemical composition of stillage	Content
Total reducing sugar (% of dry matter)	9.74 ± 0.04
Lipid (% of dry matter)	8.49 ± 0.12
Protein (% of dry matter)	58.50 ± 0.12
Ash (% of dry matter)	21.47 ± 0.20
Dry weight (% of total stillage)	11.55 ± 0.30

Effects of initial sugar concentration on lactic acid and biomass production

Effect of initial sugar concentration on lactic acid production and sugar consumption in batch fermentation is presented in Figure 1. The kinetics of sugar consumption and lactic acid production was similar for all samples (Fig. 1), but the highest percentage of theoretical lactic acid yield of 92.3 % was obtained in the sample with the lowest initial sugar concentration of 55.72 g L^{-1}. The highest lactic acid concentration of 75.06 g L^{-1} was achieved in the sample with 85.31 g L^{-1} of initial sugar. However, a lower yield (88.0 %) and productivity (1.29 g L^{-1} h^{-1}) were obtained in this sample as a result of substrate inhibition. Liu et al. (2005) as well noticed a decrease in yield with an increase of initial sugar concentrations in the fermentation performed by *Rhizopus sp*. Also, in fermentations with *Lactobacillus sp*. similar effect was spotted. Bai et al. (2003) reported that lactic acid production on chemically defined media was the most productive with initial sugar concentration of 30 g L^{-1}, and productivity declined by raising the concentrations up to 90 g L^{-1}. In Figure 1, it can be noticed that the rate of sugar consumption slightly decreased after 36 h of fermentation in samples with greater initial sugar concentration, most probably because of the increase in lactic acid concentration and thus its inhibitory effect. Similarly, Gonçalves et al. (1997) observed that ionic form of lactic acid could influence the sugar consumption at pH value maintained above 5.0.

Lactic acid can be produced on triticale, corn and wasted bread liquid stillage with yields of 0.50 g g^{-1}, 0.76 g g^{-1} and 0.80 g g^{-1}, respectively (Djukić-Vuković et al., 2011). A yield of approximately 0.92 g g^{-1} and lactic acid concentration of 50.83 g L^{-1} achieved in this study on the whole wasted bread stillage were higher than previously reported which could be explained by a positive effect of nutrients present in the whole stillage including a solid fraction. Reported lactic acid concentrations and yields obtained on many other waste

substrates were also lower than this obtained on the whole stillage. Coelho et al. (2010) reported a maximal lactic acid concentration of 41.65 g L^{-1} after 48 h of fermentation of cassava wastewater with initial sugar concentration of 50 g L^{-1}. Lactic acid concentration attained on the stillage in this study was pretty high compared to the results reported on other waste substrates, especially taking into account that the stillage was not supplemented with any nitrogen and mineral sources.

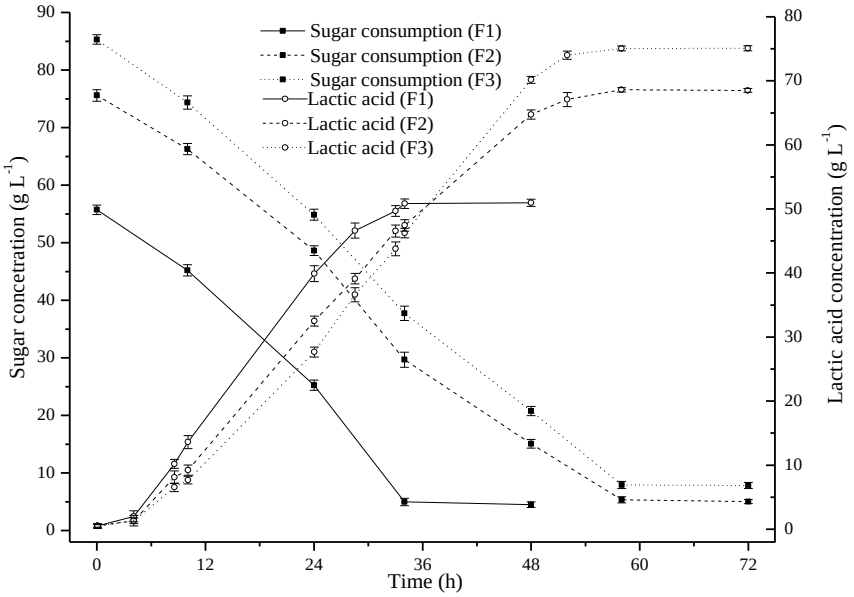

Figure 1. Lactic acid production and sugar consumption in samples with different initial sugar concentrations (F1) - 55.72 g L^{-1}; (F2) - 75.58 g L^{-1}; (F3) - 85.31 g L^{-1})

Spent fermentation media as a biomass enriched animal feed

During the batch fermentation the growth of *L. rhamnosus* ATCC 7469 was intense and it reached a number of 10^9 CFU ml^{-1} at the end of batch fermentation with initial sugar concentration of 50.83 g L^{-1}. Fermented stillage had many advantages over the non-fermented in animal nutrition. Generally, stillage was used in the form of wet distillers grains and in a dried form as Dried Distillers Grain with Solubles - DDGS in EU and USA, respectively, for animal feed (Wilkie et al., 2000). As a result of lactic acid fermentation performed by *L. rhamnosus* the stillage has been enriched with very high number of viable biomass. Scheuermann, (1993) reported improvement in nitrogen digestibility of animal feed rich in probiotic bacteria that qualifies it as a high-value animal feed. On the other hand, probiotic characteristics are strain/species specific and must be proven (Shah, 2000; Gaggia et al., 2010). Beneficial effects of probiotics depend on their survival through harsh gastric conditions of low pH in stomach. The survival of residual *L. rhamnosus* ATCC 7469 biomass of lactic acid fermentation on the stillage is presented in Figure 2. It is important to note, according to European Regulation (EC, 2008), a minimum content of viable bacterial cells (CFU kg^{-1}) in a complete feedingstuff is the one of the major specifications for animal feed enriched with probiotics. The number of *Lactobacillus sp.* and *Enterococcus sp.* cells in a healthy animal intestine amounts 10^5 - 10^8 CFU g^{-1} and their number should be about 10^9 CFU kg^{-1} in complete feed (Anadón et al., 2006; Busch et al., 2004).

Taking into account documented adherence capability of *L. rhamnosus* ATCC 7469 strain (Tuomola and Salminen, 1998) and high viable cell number in the stillage remained after lactic acid removal; it could be a promising animal feed. As shown in Fig. 2, 92.5 % of *L. rhamnosus* ATCC 7469 survive after 3 hours which corresponds to a number of viable cells of 6.6 $\times 10^8$ CFU ml^{-1}. These results are higher than those previously reported for some other *L. rhamnosus* strains (Succi et al., 2005). The pH in stomach is significantly affected by the presence of food (Gaggia et al., 2010), especially proteins which could exhibit buffering capacity. Succi et al. (2005) studied survival in MRS broth with pH adjusted to 2 and 3. Better survival in spent fermentation media in our study could be explained by higher dry

matter content, higher total protein content and presence of solid particles in media. Adaptation of some LAB to low pH affects survival after passage through stomach due to increased expression of stress protein genes (Shah, 2000). Because of the control of pH performed in 4 hours intervals in this study, some adaptation of *L. rhamnosus* ATCC 7469 cells may occur and influence on promoted survival.

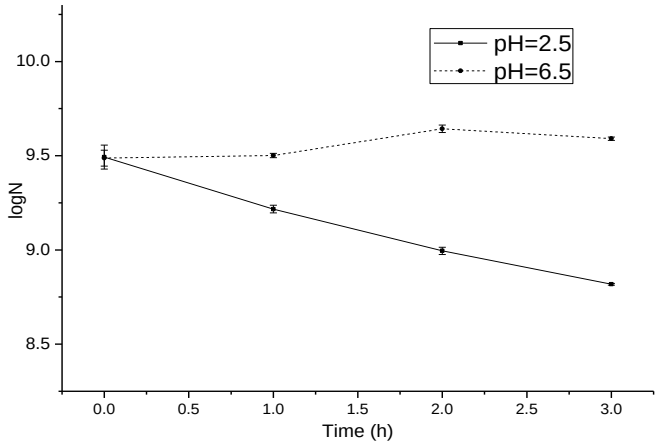

Figure 2. Survival of *L. rhamnosus* ATCC 7469 under the low pH

The European Food Safety Authority established Qualified Presumption of Safety (QPS) status which should be issued to microorganisms with proven safety and no report can be found on safety concerns related to *Lactobacillus sp.* in animals (EFSA, 2007; Gaggia et al., 2010). *L. rhamnosus* is qualified as acceptable for use in animal feed according to QPS and it is one of the most often used species in animal nutrition (Anadón et al., 2006).

CONCLUSIONS

Production of lactic acid and biomass in batch fermentation on the whole distillery stillage as a cheap and abundant substrate could be a new integrated approach to overcome ecologically important problem and improve the economy of bioethanol production. Prospective probiotic characteristics of *L. rhamnosus* ATCC 7469 strain are important for application of spent fermentation as a high-quality animal feed. Furthermore, the stillage was evaluated as a very good substrate for parallel lactic acid and biomass production since high lactic acid yields and biomass concentrations where obtained without nitrogen and mineral supplementation.

ACKNOWLEDGEMENTS

Research presented in this paper was funded by Ministry of Education and Science, Republic of Serbia, project number TR 31017.

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SELECTION OF *LACTOBACILLUS* STRAINS FOR FUNCTIONAL WHEY-BASED BEVERAGE PRODUCTION

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ABSTRACT: The aim of this study was selection of appropriate *Lactobacillus* strains for production of functional whey-based beverage. Sixteen strains of different species of *Lactobacillus* genera were studied and their abilities to carry out fast production of the beverage with satisfactory fermentation parameters were assessed.
Preliminary screening was performed by evaluating volumetric productivity of tested strains after 24 h of the fermentation. Further selection was based on determining beverage production parameters such as fermentation time, titratable acidity, cell number and sensory characteristics. The strains showed remarkable differences in cell number and sensory properties, which were particularly useful in the selection.
Based on the obtained results, the strains *Lb. casei* ssp. *casei* ATCC 27139 and *Lb. johnsonii* NRRL B-2178 are good candidates for the beverage production. These strains are rather similar in terms of beverage production ability, but strain *Lb. johnsonii* NRRL B-2178 is a top candidate for functional whey based beverage production. This strain attained titratable acidity of 9.2 °SH after 10 h of fermentation, appropriate odor and cell number of 6.8 log CFU/ml.
Key words: *whey, functional beverages, probiotics, Lactobacillus, fermentation*

INTRODUCTION

Whey which is generated in the process of cheese production is a major by-product of the dairy industry. The excess whey, with failure to processing is becoming a very big pollutant because of high COD (57-75 g/l) (Klasnja and Sciban, 2000) and BOD_5 (35-40 g/l) values (Marwaha and Kennedy, 2007; Anekar and Rao, 2009), what is completely at odds with the potential that such material posses. Discharging of the excess whey is an unforgivable loss of nutritionally valuable raw material. The global production of whey is increasing over the years and has reached the level of 178 million ton in 2010. Long-term predictions estimate that the global whey production will grow more than 2% per year until 2020 (OECD-FAO Agricultural Outlook 2010-2019, 2010), which means that its utilization must be increased. In recent years, the production and export of the whey in Serbia is almost nonexistent. Serbian export of whey has value of 10 thousands dollars, in contrast to the import which has significant value and ranges from 1.9 to 4.7 million dollars (Obućina et al., 2010).
There are many possibilities for whey utilization. In recent years, much attention has been focused on manufacturing of high value products such as lactic acid, ethanol, microbial proteins, β-D- galactosidase, vitamins (Stanzer et al., 2002) Great demands in terms of technological equipment and creating acceptable technological solutions make these processes still very expensive to implement, so most factories are limited to simpler processes of whey utilization (Affersholt, 2007). The production of functional whey based beverages, by fermentation with lactic acid bacteria (LAB), is economically most favorable way of whey processing. In this manner, within a single technological process all whey potentials can be exploited, since whey represents a high biological value material (Smithers, 2008). The role of LAB in functional whey based beverages is to assist in: (a) the preservation of the milk by the generation of lactic acid and possibly antimicrobial compounds; (b) the production of flavor compounds (e.g. acetaldehyde in yoghurt and

cheese) and other metabolites (e.g. extracellular polysaccharides) that will provide a product with the organoleptic properties desired by the consumer; (c) improving the nutritional value of food, as in, for example, the release of free amino acids or the synthesis of vitamins; and (d) the provision of special therapeutic or prophylactic properties against cancer (Reddy et al., 1973; Fernandes et al., 1987; Gilliland, 1990; O'Sullivan et al., 1992) and control of serum cholesterol levels (Lin et al., 1989). Commonly used species of LAB in production of functional whey based beverages are *Lactobacillus acidophilus, Lactobacillus delbrueckii* ssp. *bulgaricus, S. thermophilus* (Pescuma et al., 2010), *Lactobacillus reuteri, Bifidobacterium bifidum* (Hernandez-Mendoza et al., 2007), *Lactobacillus rhamnosus, Propionibacterium freudenreichii* ssp. *shermanii* (Maity et al., 2008), *Lactobacillus casei* (Dragalić et al., 2005), *Lactobacillus plantarum, Lactobacillus helveticus, Enterococcus faecium* (Pavunc et al., 2009), *Bifidobacterium animalis* ssp. *lactis* (Matijević et al., 2009) and *Lactobacillus paracasei* (Matijević et al., 2008). Large number of strains belonging to these species are not explored enough for the production of functional beverages and no information was given could it be used for the production of functional beverages. Most of the research made regarding fermented whey drink formulations was based on the use of probiotic (Drgalić et al., 2005), yogurt starters (Almeida et al., 2009; Gallardo-Escamilla et al., 2007) or kefir grains in reconstituted whey or whey and milk mixtures (Athanasiadis et al., 2004).

The aim of this study was the selection of appropriate *Lactobacillus* strains for the production of functional or probiotic whey-based beverage.

MATERIAL AND METHODS

Microorganisms and media

Sixteen strains belonging to the different species of *Lactobacillus* genera were studied. The strains used in this work (table 1) were obtained from the Collection of Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, Belgrade.

Stock cultures of lactic acid bacteria (LAB) were stored at -20°C in 3 ml vials containing MRS broth and 50% (v/v) glycerol as a cryoprotective agent. For the preparation of laboratory cultures, a drop of stock culture were transferred in 3 ml of the MRS broth and incubated for 18 h under anaerobic conditions at the optimal growth temperature (30 or 37 ºC). All working strains were pre-cultured twice in MRS broth prior to experimental use.

Whey fermentation

Sweet whey powder (Lenic Laboratories, Belgrade, Serbia), with following composition: proteins 12.11%, lipids 1.0%, and carbohydrates 69.62%, was reconstituted in water to contain 8% of dry matter. A volume of 100 ml of the reconstituted whey with pH value 6.2 was poured into sterile glass bottles of 200 ml. Samples were pasteurized at 60 °C for 60 min, cooled at fermentation temperature and immediately inoculated by adding 2% (v/v) of 18 h - old testing cultures grown in MRS broth. All fermentations were performed at 37 ºC, statically. In the experiment of preliminary screening, the samples were incubated for 24 h. Subsequently, the volumetric productivity (P) was determined. In the experiment of evaluation of beverage production performances, fermentation was carried out until pH=4.6 was attained. During the incubation time, the samples were withdrawn every 1 h for determination of pH value. By the time when pH=4.6 was reached, fermentation was stopped by quick cooling. Beverage production performances of tested strains were evaluated by determining the basic fermentation parameters such as fermentation time (h), titratable acidity (°SH), cell number (log (CFU/ml)) and sensory characteristics.

Analytical methods

The volumetric productivity and titratable acidity were determined on the basis of produced lactic acid, by titration of 20 ml of whey samples with 0.1 M NaOH to pH=8.2 in the presence of phenolphthalein as the indicator. Titratable acidity was expressed in Soxhlet-Henkel degrees (°SH), while the volumetric productivity was expressed in g/l·h. These parameters were calculated by the following equation:

$$^{\circ}SH = a \cdot f_{NaOH} \cdot 2$$
$$g/l \cdot h_{(lactic\ acid)} = {}^{\circ}SH \cdot 0.225 / 24$$
$$a = ml\ 0.1\ M\ NaOH$$
$$f = titration\ correction\ factor\ of\ 0.1\ M\ NaOH$$

Cell number was determined by pour plate counting method on MRS agar and expressed in log (CFU/ml). For determination of sensory characteristics a panel group of 5 sensory analysts were evaluating odor with 1-5 grades according to fallowing scale: 1-on sauerkraut, 2-on sourdough, 3-on whey, 4-on mild yogurt, 5-on yogurt.

RESULTS AND DISCUSSION

Preliminary screening based on fermentation activity of tested strains

The fermentation activity of sixteen tested strains was investigated. The fermentation activity was evaluated by determining the volumetric productivity of tested strains. The results are presented in table 1.

Table 1. Volumetric productivities (P) obtained after 24 h of whey fermentation with different strains of *Lactobacillus* sp.

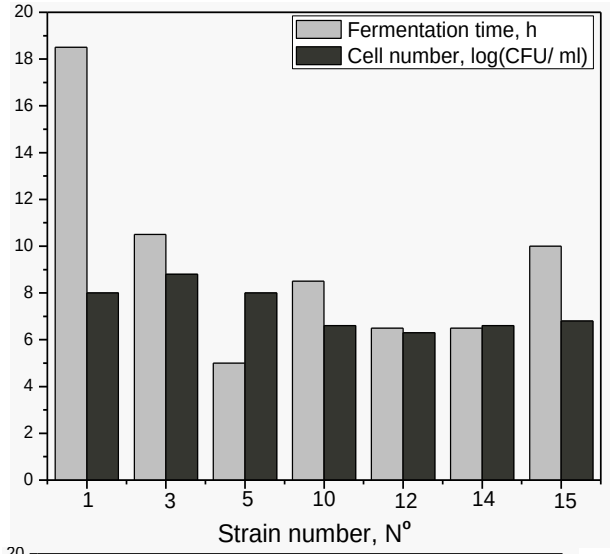
Strain N°	Strains of *Lactobacillus* sp.	P(g/(l·h))
1	*Lb. gasseri* NRRL B-14168	0.124
2	*Lb. gasseri* NRRL B-4240	0.059
3	*Lb. casei* ssp.*casei* ATCC 27139	0.262
4	*Lb. casei* ssp. *casei* NRRL B-441	0.244
5	*Lb. helveticus* ATCC 15009	0.358
6	*Lb. helveticus* NRRL B-734	0.088
7	*Lb. reuteri* ATCC 23272	0.123
8	*Lb. paracasei* ssp. *paracasei* NRRL B-4564	0.083
9	*Lb. rhamnosus* TM1	0.239
10	*Lb. rhamnosus* ATCC 7469	0.280
11	*Lb. acidophilus antibiophilus*	0.262
12	*Lb. acidophilus* ATCC 4356	0.267
13	*Lb. delbrueckii* ssp. *lactis* NRRL B-1942	0.097
14	*Lb. delbrueckii* ssp. *lactis* NRRL B-4525	0.341
15	*Lb. johnsonii* NRRL B-2178	0.207
16	*Lb. delbrueckii* ssp. *bulgaricus* ATCC 11842	0.171

As shown in table 1, the majority of strains (62.5 %) showed an acceptable level of fermentation activity. The highest productivity of 0.358 g/l·h was obtained in fermentation by the strain *Lb. helveticus* ATCC 15009, while the lowest productivity of 0.059 g/l·h was obtained by the strain *Lb. gasseri* NRRL B-4240.

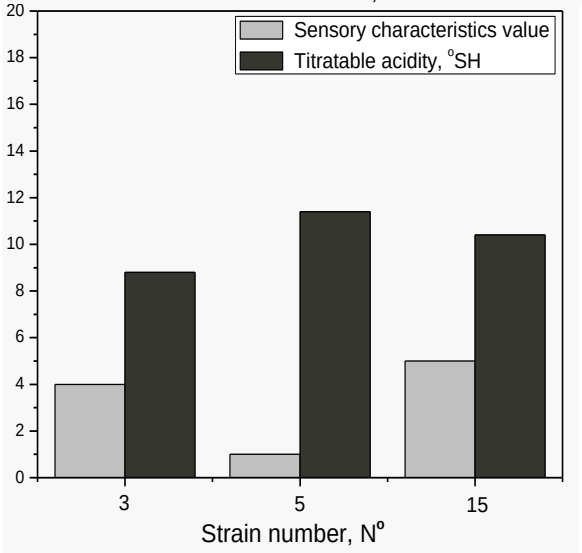
Based on the obtained results, one strain of each species was selected for further testing of the production performances. The preliminary selected strains were *Lb. gasseri* NRRL B-14168, *Lb. casei* ssp. *casei* ATCC 27139, *Lb. helveticus* ATCC 15009, *Lb. rhamnosus* ATCC 7469, *Lb. acidophilus* ATCC-4356, *Lb. delbrueckii* ssp. *lactis* NRRL B-4525, *Lb. johnsonii* NRRL B-2178.

Selection based on beverage production performances

After the preliminary screening, the beverage production performances were investigated. Seven pre-selected strains were characterized on the basis of fermentation parameters which were important in the production of functional beverages.
Time of fermentation was one of the most important parameters which could substantially decrease the beverage production costs and thus valorize whey from the cheese production. In addition, dairy products should contain ≥ 6 log CFU/ml of viable probiotic bacteria to induce beneficial effects on health what is a prerequisite for regular consumption (Tamime et al., 1995; Kailasapathy and Rybka, 1997). Since whey itself has not an acceptable taste, the sensory characteristics are an essential parameter for inclusion of produced beverage in a human consumption. Fig.1-a compares fermentation time and cell number achieved during the fermentation with pre-selected strains. Fig.1-b compares values of sensory characteristics and titratable acidity achieved with three selected strains during the fermentation.



(a) Fermentation time and cell number achieved with pre-selected strains during whey fermentation.



(b) Sensory characteristic values and titratable acidity achieved with three selected strains during whey fermentation

Figure 1. Beverage production performances of strains belong to *Lactobacillus* genera. Strain Number, N° is presented in tab 1.

As shown in Fig.1-a, short fermentation time (5-6.5 h) was obtained with strains *Lb. helveticus* ATCC 15009, *Lb. acidophilus* ATCC-4356 and *Lb. delbrueckii* ssp. *lactis* NRRL B-

4525, but only *Lb. helveticus* ATCC 15009 achieved high cell number of 8.0 log CFU/ml. Strain *Lb. rhamnosus* ATCC 7469 achieved quite short fermentation time (8.5 h) but a cell number of 6.6 log CFU/ml. On the other hand, strains *Lb. casei* ssp. *casei* ATCC 27139 and *Lb. johnsonii* NRRL B-2178 had quite longer fermentation time (10.5 and 10.0 h, respectively) and achieved cell numbers of 8.8 log CFU/ml and 6.8 log CFU/ml, respectively. Strain *Lb. gasseri* NRRL B-14168 had too long fermentation time of 18 h. It is interesting to note that the strain *Lb. delbrueckii* ssp. *lactis* NRRL B-4525 which exhibited the best volumetric productivity and short time of fermentation, didn't achieve the highest cell number at the end of fermentation. This could be due to differences in lactic acid production abilities and growth characteristics. Strains *Lb. casei* ssp. *casei* ATCC 27139, *Lb. helveticus* ATCC 15009 and *Lb. johnsonii* NRRL B-2178 were selected as most promising. These strains were further characterized based on the sensory characteristics and titratable acidity and results are presented in Fig. 1-b.

As shown in Fig.1-b, the strain *Lb. helveticus* ATCC 15009 exhibited very low grade for odor which was like sauerkraut. This is probably a consequence of unpleasant odor substances produced by this strain. This strain was therefore rejected as a possible producer of the beverages. Strains *Lb. casei* ssp. *casei* ATCC 27139 and *Lb. johnsonii* NRRL B-2178 achieved higher grades (4 and 5, respectivly) for odour than the strain *Lb. helveticus* ATCC 15009. These two strains achieved titratable acidity of 8.8 and 9.2 °SH, respectivly and they are rather similar in terms of beverage production ability. The strain *Lb. johnsonii* NRRL B-2178 is a top candidate for production of functional whey based beverage due to much stronger odor like yogurth which was evaluated with grade 5. It is also interesting to note that the strain *Lb. johnsonii* NRRL B-2178 has probiotic characteristics and shows high viability in the presence of whey proteins during the storage (EL-Shafei et al., 2010), which is essential for the beverage shelf-life. In addition, all strains produced residue-free beverages with the sour-salty taste beacuse of high amount of present lactic acid.

CONCLUSIONS

Fermentation of whey by LAB could be an interesting alternative for the production of functional beverages. Our study showed that the strains *Lb. casei* ssp. *casei* ATCC 27139 and *Lb. johnsonii* NRRL B-2178 are good candidates for the beverage production. The strain *Lb. johnsonii* NRRL B-2178, out of 16 tested strains, is a top candidate for the functional whey based beverage production. This strain attained titratable acidity of 9.2 °SH after 10 h of fermentation, appropriate odor and cell number of 6.8 log CFU/ml.

ACKNOWLEDGEMENTS

This work was funded by the Serbian Ministry of Education and Science (TR 31017).

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SUGAR CONTENT CHANGES DURING MILK FERMENTATION WITH KOMBUCHA ADDITION

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ABSTRACT: The aim of this research was to examine changes in sugar content during production of fermented milk beverages from milk with 2g/100g fat by using 10% (v/v) inoculum of kombucha, cultivated on black tea. Kombucha is a mixed culture of acetic acid bacteria and yeasts with complex metabolic pathways. The traditional carbon source for kombucha fermentation is sucrose. Novel researches showed that application of any other sugar such as lactose is possible.
Temperatures for milk fermentation were 37°C and 42°C. Final pH value was 4.6. Dry matter, milk fat, total proteins and ash were analysed in milk and products by standard methods. Changes in lactose, galactose, glucose and fructose content were investigated during fermentation on pH 5.8; 5.4; 5.1; 4.8 and 4.6. Lactose, galactose, glucose and fructose were detected using specific enzymatic tests.
The obtained results indicated that the pattern of changes of lactose, galactose, glucose and fructose concentration during milk fermentation on two different temperatures were similar.
Key words: *Kombucha, milk fermentation, sugar content*

INTRODUCTION

Kombucha is well-known association of several yeast species and acetic acid bacteria, its metabolic activity on tea sweetened with sucrose produces a pleasant refreshing beverage, containing a number of useful compounds (Malbaša et al., 2008). Previous investigations (Markov et al., 2001) have shown that the applied fungus contained at least five yeast strains (*Saccharomycodes ludwigii, Saccharomyces cerevisiae, Saccharomyces bisporus, Torulopsis* sp. and *Zygosaccharomyces* sp.) and two bacterial strains of the genera *Acetobacter*.
Reiss (1994) investigated application of other sugars (lactose, glucose or fructose) for kombucha cultivation. He has found that kombucha fermentation on other sugars produced beverages slightly different in flavour but significantly different in ethanol and lactic acid quantity, compared to sucrose's sweetened tea. For example, fermentation on lactose gave extremely low quantities of ethanol in comparison to the fermentation on sucrose.
Fermentation is one of the oldest methods practiced by human beings for the transformation of milk into shelf life products. Around 400 generic names are applied to the traditional and industrial fermented milk products manufactured throughout the world (Kurmann et al., 1992). Milk has a very complex structure even though its constituents are mainly water, lactose, fat, proteins and minerals. In cows' milk, milk non fat solid level is 8.5–9.0 g/100g, of which around 4.5 g/100g is lactose, 3.3 g/100g protein (2.6 g/100g casein and 0.7 g/100g whey proteins), and 0.7 g/100g mineral salts. Each of these components is important for the production of a satisfactory yoghurt quality (Fuquay et al., 2011). Nutritional value of fermented dairy products consists, as in milk, in primary essentially ingredients necessary for human body growth, reproduction, maintenance, renewal and fulfill energy needs. These products contain, as well as milk, lactose, lactic acid, protein, fat, minerals and vitamins. The concentration of mentioned components in yoghurt mainly depends of applied starter cultures.

Lactose is the main carbohydrate in dairy products. It contributes 30% energy of whole cow's milk. This disaccharide is composed of glucose and galactose and is the only saccharide synthesized by mammals (Smolin and Grosvenor, 2000). Lactose plays an important role in the formation of the neural system and the growth of skin (texture), bone skeleton and cartilage in infants. It also prevents rickets and saprodontia (Emmett and Rogers, 1997). Lactose fermentation by lactic acid bacteria to lactic acid is widespread in the production of fermented dairy products. In particular, lactose is the principal energy source for the bacteria in the starter culture, while casein, together with calcium and phosphorus gives rise to the basic structure of a gel structure (John W. Fuquay et al., 2011). In general, dairy starter cultures metabolize lactose either through the homo- or hetero-fermentative metabolic pathways. *S. thermophilus*, L. *delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* possess β-galactosidase (EC 3.2.1.23.) to hydrolyze lactose and fermentation is homofermentative type. Under the influence of lactic acid bacteria, glucose is converted into lactic acid, and alternatively, into ethanol and CO_2 if yeasts are present (Figure 1). Lactose content is reduced during fermentation (by 20–30 g/100g of the level in the original milk), while the concentration of lactic acid and some free amino acids increases, for example, proline, serine, alanine, valine, leucine, and histidine. Even though, after fermentation, the product may contain 4-5g /100g of lactose (Tamime and Robinson, 2004)

Lactose hydrolysis in milk and other dairy products by the enzyme galactosidase is of considerable interest to the dairy industry. It has been estimated that over 70% of world's inhabitants suffer from either lactose maldigestion or intolerance (De Vrese et al., 2001.) Most people who can not consume milk due to allergies on lactose or protein, consume yoghurt and therefore provide the balance of microflora in an intestinal tract.

Application of kombucha in lactose fermentation is still under investigations. Therefore the aim of this research was to compare the changes in lactose, galactose, glucose and fructose content during milk fermentation by non conventional starter-kombucha, at pH 5.8; 5.4; 5.1; 4.8 and 4.6, at two different temperatures 37°C and 42°C.

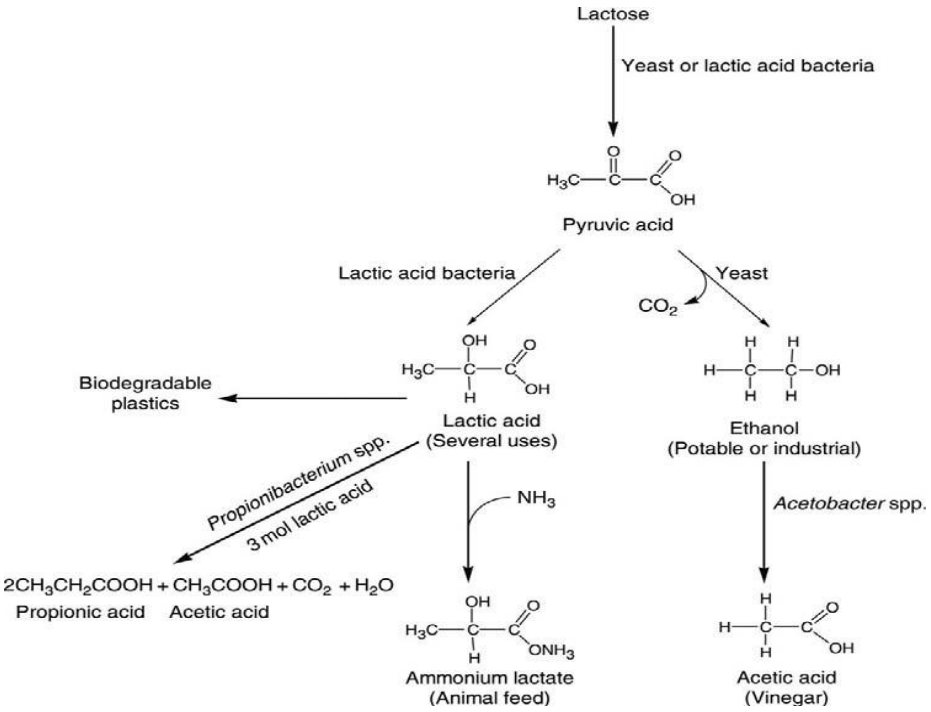

Figure 1. Fermentation products from lactose (Fuquay et al., 2011)

MATERIAL AND METHODS

Milk
Homogenized and pasteurized cow's milk from AD Imlek, Division Novi Sad Dairy, was used for the production of kombucha fermented milk products. The composition of milk was as follows: fat content – 2.0 g/100g, total solids – 10.59 g/100g, total proteins – 3.30 g/100g and lactose – 4.60 g/100g, ash 0.69 g/100g.

Kombucha inoculums
Kombucha inoculum was prepared according to procedure published by Malbaša *et al.,* (2009). Kombucha is cultivated on a black tea (*Camellia sinensis* – oxidized, 1.5 g/L) with sucrose concentration of 70 g/L. The tea was cooled to room temperature, after which inoculum from a previous fermentation was added in concentration of 10 mL/100mL. Incubation was performed at 25°C for 7 days. Inoculum in concentration of 10 mL/100mL was used for milk inoculation.

Samples production
Samples were obtained by addition of 30 mL of kombucha inoculum in 300 mL of milk at 42°C and 37°C simultaneously. Samples were taken at pH values: 5.8; 5.4; 5.1; 4.8 and 4.6.

Methods
Chemical composition: dry matter, milk fat, total proteins and ash of samples was analyzed by methods according to Carić et al. (2000).
The content of lactose and products of milk fermentation at two temperatures were detected in all samples using specific enzymatic tests, such as K-FRUGL 11/05 (D-glucose and D-fructose) and K-LACGAR 12/05 (lactose and D-galactose). The analyses were performed according to the guidelines supplied by the producer (Megazyme, Ireland). Products of the reactions were monitored using spectrophotometer (T80+UV/VIS Spectrometer PG Instruments Ltd.).
Statistical analysis of results was carried out with the computer software program "Statistica 9" and results were expressed as average values with standard deviation.

RESULTS AND DISCUSSION

The obtained results indicate no significant differences in dry matter, fat, proteins and ash content in samples produced at 37°C and 42°C (Table 1).

Table 1. Chemical composition changes in during milk fermentation

	42° C		37° C	
Components (g/100g)	**pH 6.07**	**pH 4.6**	**pH 6.07**	**pH 4.6**
Dry matter	10.34 ±0.02	10.34±0.03	10.34±0.02	9.98±0.24
Milk fat	2.00±0.00	1.98±0.00	2.00±0.00	1.98±0.00
Proteins	3.12±0.12	3.14±0.08	3.12±0.12	2.82±0.17
Ash	0.69±0.69	0.68±0.01	0.69±0.69	0.62±0.05

The biggest changes happened on lactose content. At the beginning of the process lactose content is 4.3900g/100g and further during fermentation it decreased continually. The drop in lactose content was notable between first and second fermentation point at the both temperatures. Lactose content was lower in samples produced at 37°C (3.5931g/100g) than in samples produced at 42°C (3.7483 g/100g) on the end of process - pH 4.6 (Table 2 and Table 3). This could be explained by longer fermentation time for samples produced on 37°C and therefore followed-up with further degradation of lactose.

Table 2. The changes of sugars content in samples obtained at 42°C

pH value

Sugar content (g/100g)	6.07	5.8	5.4	5.1	4.8	4.6
Lactose	4.3900± 0.000	3.9607± 0.0279	3.7910± 0.0080	3.7826± 0.0558	3.7526± 0.0652	3.7483± 0.0254
Galactose	0.0068± 0.0045	0.2017± 0.0075	0.2760± 0.0137	0.2793± 0.0057	0.2890± 0.0095	0.2576± 0.0040
Glucose	0.0155± 0.0155	0.1460± 0.0030	0.1527± 0.0056	0.1536± 0.0020	0.1326± 0.0041	0.0840± 0.0093
Fructose	0.0125± 0.0125	0.0276± 0.0276	0.0232± 0.0034	0.0109± 0.0037	0.0129± 0.0001	0.0233± 0.0035

The galactose and glucose content increased between first and second pH point is caused by lactose fermentation and its hydrolyses on glucose and galactose. Contents of these compounds decreased as consequence of starter culture metabolic activity during fermentation. These results are in accordance with previous research (Iličić et al., 2009). The fructose content at pH 4.6 was higher in samples produced at 42°C compared with samples produced at 37°C, that revealed kombuha's higher fructose affinity at lower temperature.

Table 3. The changes of sugars content in samples obtained at 37°C

	pH value					
Sugar content (g/100g)	**6.07**	**5.8**	**5.4**	**5.1**	**4.8**	**4.6**
Lactose	4.3900± 0.000	3.8523± 0.0881	3.8210± 0.0478	3.7713± 00563	3.6105± 0.1006	3.5931± 0.1388
Galactose	0.0068± 0.0045	0.4097± 0.0534	0.3801± 0.0344	0.3947± 0.0213	0.3272± 0.0295	0.3188± 0.1863
Glucose	0.0155± 0.0155	0.3371± 0.1384	0.2206± 0.0388	0.1948± 0.0518	0.1318± 0.0186	0.0817± 0.041
Fructose	0.0125± 0.0125	0.0964± 0.0145	0.0762± 0.0234	0.1043± 0.0066	0.0752± 0.0077	0.0865± 0.0057

CONCLUSIONS

The changes pattern of lactose, galactose, glucose and fructose concentration during milk fermentation on two different temperatures were similar. The highest increase of glucose, fructose and galactose content was between pH 6.07 and pH 5.8, as well as lactose decrease. Lower lactose and fructose content at pH 4.6 was obtained in samples produced at 37°C compared with samples produced at 42°C.

ACKNOWLEDGEMENTS

Authors want to thank Ministry of Education and Science of Republic of Serbia for the financial support of investigations presented in this article, Project No. 46009.

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SYNTHESIS OF ETHYL CINNAMATE CATALYZED BY LIPASE B FROM *CANDIDA ANTARCTICA*

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ABSTRACT: Cinnamic acid and its esters are widespread throughout plant kingdom and therefore, they are common components of our daily diet. Ethyl cinnamate has been approved by FDA for direct addition to food for human consumption. Enzyme synthesis is strongly preferred compared to chemically catalyzed esters synthesis when product quality is a main issue, as is the case for food production. The aim of this study was to examine possibilities for enzyme catalyzed synthesis of cinnamic acid esters and to optimize the synthesis of ethyl cinnamate in terms of selected parameters, including the type of the organic solvent and substrate initial molar ratio.
All reactions were performed batchwise under pH and temperature control in vessels containing 5 mL of reaction medium, using cinnamic acid as limiting substrate (0.167 M). Each reaction mixture was supplemented with 75 mg of commercial immobilized lipase B from *Candida antarctica*, Novozyme 435. The antioxidant activity of obtained ester was measured by using chemical and electrochemical techniques.
We have proven that ethyl cinnamate can be synthesized using lipase B from *Candida antarctica* with very high reaction yields in the simple bioreactor system.The best reaction yield (89%) was obtained in isooctane when substrate molar ratio of 1:3 was used. Both, DPPH assay and cyclic voltammetry measurement has shown that ethyl cinnamate has better antioxidant properties than cinnamic acid itself.
Key words: *Cinnamic acid, Ethyl cinnamate, Enzymatic esterification, Radical-scavenging activity, Antioxidant activity, Cyclic voltammetry*

INTRODUCTION

Phenolic acids, especially cinnamic acid and its derivatives, are inevitable component of our daily diet due to their ubiquitous presence in plant food. These compounds are components of soybeans, cotton seeds, peanuts, coffee and they are proven to be one of the reasons for antioxidant potential of oilseeds (Stamatis et al., 1999). Consequently, these phenolic acids are very interesting substrates for the production of a variety flavor and fragrance compounds such as ethyl cinnamate. There is growing interest in cinnamic acid esters synthesis using free or immobilized lipase in non-aqueous solvents. Although their synthesis can also be chemically catalyzed by acids or bases, enzymatic esterification offers environmental advantages and a reduction in energy consumption. Furthermore, the enzyme synthesis is strongly preferred when product quality is a main issue as is the case for food production.
Guyot and coworkers were first to report enzymatic esterification of phenolic acids and fatty alcohols catalyzed with lipase B from *Candida antarctica* (Guyot et al., 1997; Buisman et al., 1998). Soon afterwards, Stamatis and his research team described enzymatic esterification of cinnamic acid with aliphatic alcohols using lipase from *Rhizomucor miehei* as the catalyst. However data related to enzyme catalyzed esterification of cinnamic acid and its esters are very incomplete and contradictory, making them an interesting topic for research.

Focus of this work was to examine opportunities for lipase catalyzed esterification of cinnamic acid with different aliphatic alcohols and to optimize ester production using ethyl cinnamate as a model reaction. Commercial enyzme preparation of lipase B from *C. antarctica* immobilized on macro porous acrylic resin was used in all experiments. This work also describes attempt to examine antioxidant properties of cinnmaic acid and its esters, thus looking into influence of esterification on antioxidant properties of cinnamic acid.

MATERIAL AND METHODS

Synthesis were performed using commercial immobilized lipase B from *Candida antarctica*, Novozyme 435, obtained from Novo Nordisk, Denmark. Cinnamic acid and alcohols, of analytical grade, were purchased from Sigma Aldrich (*St Louis, MO*, USA), while isooctane and other solvents used in experiments were purchased from Fluka (StQuentin-Fallavier, Switzerland).

Esterification of cinnamic acid

The esterification reactions were carried out batchwise at 55 ºC and 250 rpm in sealed, stirred flasks (100 mL). Typically, reaction mixture consisted of 0.846 mmol of cinnamic acid, and 15 equivalents of alcohol (C2, C4-C7), and was filled with isooctane to reach a total volume of 5 mL. Each reaction mixture was supplemented with 75 mg of enzyme. Control reactions, without enzyme, were performed parallely under the same conditions.

Optimization of parameters for ethyl cinnamte production

In order to maximize production of ethyl cinnamate, series of experiments were performed, in which type of organic solvent and substarte initial molar ratios (1:1, 1:3, 1:5, 1:7, 1:9, 1:12, 1:15, and 1:20) were alternatively varied. The effect of solvent polarity on product formation was examined using solvents with different log*P* values.

Analysis and monitoring of reaction mixtures

Ester formation was monitored by titration of residual cinnamic acid with NaOH (0.05M) in presence of phenolphthalein as indicator. Reaction yields were calculated simply with respect to the limiting reagent (cinnamic acid or its derivatives) by dividing amount of the substrate that was consumed in reaction with the initial amount of substrate.

Antioxidative activity measured by DPPH assay and cyclic voltammetry

Antioxidative activity of cinnamic acid and its esters was measured by their ability to scavenge DPPH radical, which was monitored by decrease of absorbance on 517 nm, as described elsewhere (Blois, 1958). A volume of 200 µL of metanolic ester solution was mixed, in spectrophotometric cuvet, with 1800 µL of metanolic DPPH solution (0.1 mM), vortexed and left in dark and after 30 min absorbance was measured at 517 nm. Appropriate ethyl cinnamate dilutions in methanol were experimentally found in order to satisfy the linear dependence of ester concentration *vs* absorbance. Calculations were done according to method described elsewhere (Gorjanović et al., 2010).

Cyclic voltammetry experiments were performed in a three electrode cell, at ambient temperature. Glassy carbon electrode was used as working electrode, Pt wire as counter, while saturated calomel electrode (SCE) served as reference electrode. The cyclic voltammograms were recorded at scan rate of 50 mV s^{-1}, starting at open circuit potential to potential of 2.0 V (for cinnamic acid) and 1.2 V (for *p*-coumaric acid and ethyl cinnamate). The experiments were performed using SP-200 (BioLogic Science Instruments) potentiostat/galvanostat.

RESULTS AND DISCUSSIONS

Effect of alcohol type on esterification of cinnamic acid

Investigation of the influence of an acyl acceptor on lipase-catalyzed synthesis of cinnamic acid esters was done. For this purposes, cinnamic acid was esterified under the same conditions using aliphatic alcohols of increasing carbon chain lengths: ethanol, *n*-butanol, *n*-pentanol, *n*-hexanol and *n*-heptanol. This assay demonstrated that all of the used alcohols could form esters with cinnamic acid under previously described conditions. Results obtained during this experiment are presented in Table 1.

Table 1. Esterification yields, reaction rates and relative reaction rates obtained with different aliphatic alcohols

Alcohol (acyl acceptore) type	Ester yield, %	Standard deviation, %	Reaction rate, v (mmol/(h cm^3)) ·10^4	Relative reaction rate, v/v_m [a]
ethanol	31	3.4	7.2	1
n-butanol	19	5.2	5.3	0.74
n-pentanol	21	0.8	4.8	0.67
n-hexanol	19	1.2	4.4	0.61
n-heptanol	16	0.4	3.8	0.53

[a]*Relative reaction rate: rection rate normalized to that obtained with optimal acyl acceptore,* v_m

A series of esterifications with alcohol of increasing chain length resulted in a decrease in ester yield, probably because of alcohols react with the acyl-enzyme intermediate formed during the acylation process, the rate of synthesis is determined by the diffusion of alcohol molecules into the active site of the enzyme. Since smaller alcohols are able to diffuse into the active site more readily than bulky ones, an increase of the chain length leads to a decrease of the enzyme activity. Ethanol gave highest reaction yield (31%). This result was somewhat unexpected because literature data showed that esterification of cinnamic acid with ethanol gave very low reaction yields (Guyot et al., 1997). Though results similar with ours were reported for synthesis of ethyl ferulate (Lee et al., 2006). Different amount of ethanol used in esterifications is probably cause of these contradictory results in literature. In fact ethanol in great excess may cause inactivation of enzyme thus making the cinnamic acid/ethanol molar ratio very important parameter in ester formation.

Effect of solvent polarity

Enzyme performances are known to be highly dependent on the properties of the organic solvent. Thus, esterification of cinnamic acid with ethanol was conducted in variety of solvents in order to determine optimal solvent for this reaction. Results obtained in this investigation are presented in Table 2.

Isooctane was proven to be the best solvent for esterification of cinnamic acid with ethanol, as can be seen from Table 2. This result agrees with literature, where has been noted that solvents with log$P$ > 4 are good for biocatalysis since they do not remove essential water layer around the enzyme (Fu and Vasduven, 2010). Conversions up to 85% within 5 days were reported for esterification of cinnamic acid with *n*-butanol, when apolar solvent *n*-pentane was used (Buisman et al., 1998). In solvents with low log$P$ values, that is in polar environments the best results are obtained when *tert*-butanol is used. This result is in accordance with the literature data, where relatively high reaction yields, 52%, have been obtained during esterification of cinnamic acid with *n*-octanol when *tert*-butanol has been used as solvent (Stamatis et al., 1999). This result is probably due to a good solubility of cinnamic acid in polar solvents.

Table 2. Effect of organic solvent on the esterification of cinnamic acid with ethanol catalyzed by immobilized lipase B from *Candida antarctica* at 55 ^{o}C and 250 rpm

Solvent	acetone	*tert*-butanol	chloroform	hexane	heptane	isooctane	hexadecane
log*P*	-0.23	0.35	2	3.5	4	4.5	8.8
Ester yield, %	traces	20	traces	22	23	31	6

Effect of initial molar ratio on reaction yield

Initial molar ratio of substrates is shown to be one of the most important parameters in enzyme catalyzed ester synthesis. Effect of this parameter on the rate of product formation was examined in isooctane, the solvent that was proven to be the best for synthesis of ethyl cinnamate. Increase in initial molar ratio (acid to alcohol) from 1:3 to 1:5 led to decrease in reaction yield for 45%, indicating that ethanol in great excess indeed deactivated enzyme. Further increase in molar ratio did not have such significant effect on product formation.

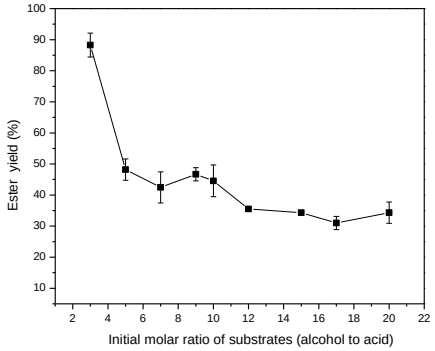


Figure 1. Effect of the initial molar ratio of substrates (alcohol to acid) on ester yield. Reaction mixture consisted of 0.0846 mmol cinnamic acid and alcohol varied according to experimental plan

DPPH assay and cyclic voltammetry results

Cinnamic acid and ethyl cinnamate were tested for their radical-scavenging activity against DPPH in order to elucidate the influence of esterification on the antioxidant activity. It appeared, as showen in Figure 2, that ethyl cinnamate has better antioxidant potential (30% for 0.167M methanolic solution) than cinnamic acid itself (4%), though it is still very low comparing to other natural phenolic antioxidants, such as gallic acid.

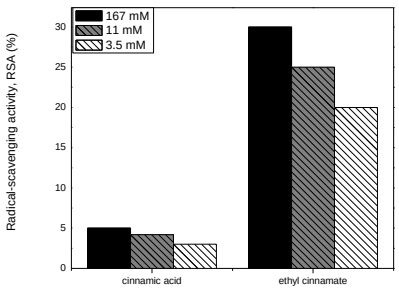
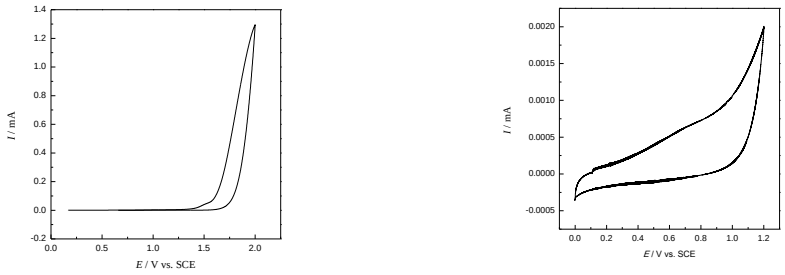


Figure 2. Free radical- scavenging activity of cinnamic acid and ethyl cinnamate

The electrochemical behavior of ethyl cinnamate was investigated by cyclic voltammetry at a scan rate of 50 mV s^{-1} in acetate buffer pH 5.6 on glassy carbon electrode and compared to

native cinnamic acid. It seems that the esterification of cinnamic acid increased its antioxidant activity (Fig 3). It was possible to observe some oxidation process in the cyclic voltammogram of ethyl cinnamate leading to wave broadening compared with that of cinnamic acid as well as appearing of minor anodic peak at E_a 0.7 V.



a) Cinnamic acid (1 mM) in sodium acetate buffer (0.2M)

b) Ethyl cinnamate (1 mM) in sodium acetate buffer (0.2M)

Figure 3. Cyclic voltammetry plots

CONCLUSION

The esterification activity of the commercial immobilized lipase towards cinnamic acids was assayed in different solvents. Isooctane is shown to be the best solvent for this reaction even though solubility of cinnamic acid in this a polar solvent is very low. Highest esterification yield of ethyl cinnamate is obtained when initial molar ratio of substrates 1:3 (cinnamic acid is limiting substrate) is used.

Esterification of cinnamic acid appeared to result in increasing radical-scavenging ability. The effect of esterification of cinnamic acid was also confirmed by electrochemical method using ethyl cinnamate which appeared to enhance the antioxidant activity. These findings should stimulate the application of such lipase-catalyzed reactions for the preparation of food acceptable esters of cinnamic acid as potential lipophilic antioxidants.

ACKNOWLEDGEMENTS

This work was supported by Grant numbers E!6750 and III 46010 from the Ministry of the Education and Science, Republic of Serbia.

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THE EFFECT OF FERMENTATION CONDITIONS ON POLYPHENOL CONTENT OF RASPBERRY WINE

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ABSTRACT: In the two past decades, there has been a great deal of research on polyphenol compounds and their antioxidant properties. Raspberry as a fruit species is widely grown in Serbia and is well known in the world for its quality. Being very rich in polyphenol compounds, raspberry is a great source of components of excessive biological value.
The objective of this study was to investigate influence of temperature, sulfur-dioxide and selected yeast on fermentation of raspberry pulp and the total polyphenol content of raspberry wine. The pulp of *Meeker*, one of the most abundant varieties of raspberry (*Rubus idaeus*) in Serbia, was fermented at 15°C and 22°C with and without selected yeast cells, and with and without addition of sulfur-dioxide. The total polyphenol content was determined every 24 hours, according to the Folin Ciocalteau method and results were expressed in mg/L gallic acid equivalents (mg GAE/L).
The obtained results indicate that fermentation temperature and addition of sulfur-dioxide significantly influence the extraction of phenolic compounds from raspberry pulp, while the addition of selected yeast cells has no influence. Immediately before fermentation, total polyphenol content in the sample was 1415,7 mg GAE/L. After 72 hours of fermentation, the sample fermented at 15°C with addition of sulfur-dioxide and without selected yeast cells reached its maximum value of 2191 mg GAE/L, whereas the same sample fermented at 22°C reached the value of 2820 mg GAE/L. Other samples had lower values then these two. After 96 and 120 hours of fermentation, in all samples total polyphenol content was slightly lower then after 72 hours.
Key words: *raspberry, fermentation, polyphenol content, raspberry wine*

INTRODUCTION

The production of wine from berry fruits has increased over the last several years in contrast to apples and pears which have been used for wine production since eighteen century. Raspberries, *Rubus idaeus* L*.,* present great source of polyphenolic compounds, particularly flavonoids such as anthocyanin pigments, which give raspberries their characteristic color (Liu, 2007). The phytochemicals such as p-coumaric, ferulic and ellagic acids, are commonly found in raspberries and may have a significant antioxidant and anticancer activity and act as a protector against biological oxidative stress in human cells (Hakkinen et al., 1999). In recent years, increased interest in human health, nutrition and disease prevention has enlarged consumers' demand for functional foods including fruits and their products such as wine (Rupasinghe and Clegg, 2006). Besides polyphenolic compounds, raspberry (cv *Meeke*r) displays specific acid and sugar contents (pH 3.2-3.6 and about 14.5° Brix) that makes it suitable for fruit wine (Duarte et al., 2010). The "*Meeker*" raspberry variety is popular due to high yields, a long harvest season, resistance to root rot, and machine harvest characteristics. "*Meeker*" fruit has a desirable color, firm texture, and good sensorial attributes including aroma, sweetness, and acidity. Raspberry fruits which cannot be used for consumption are used in the production of juices, jam, sweets etc. In some regions, raspberry producers are looking for new alternatives for the use of small and crushed raspberry fruits. One of them is wine (Duarte et al. 2010). Besides polyphenol compounds, the concentration of certain minerals in wine is important due to health impact of minerals on the human organism, their role in the stability of wine, and food regulations. Generally, fruit wines, among which is raspberry wine, contain a significant amount of polyphenols. Range of polyphenols in fruit wines is from 335 mg/L GAE for strawberry to 1830 mg/L GAE for

black currant. Raspberry wine is between these values with total polyphenol content of 1050 mg of GAE/L, which puts it among the top five according to the content of polyphenols in fruit wines (Heinonen et al., 1998).
The objective of this study was to investigate the influence of fermentation conditions on the total polyphenol content and antioxidative power of raspberry wine. Eight samples of the same raspberry pulp was subjected to fermentation at different conditions. Total polyphenol content was measured daily from the beginning until the end of fermentation to obtain the overall picture of polyphenol extraction during fermentation.

MATERIAL AND METHODS

Materials and reagents

Raspberry fruits of the Meeker variety were obtained from the farm in the vicinity of Aleksandrovac town, Serbia. Fruits were stored at -25°C under the processing. The selected yeast, *Saccharomyces cerevisiae bayanus* EC-1118 was purchased from Lallemand (Montreal, Canada). Reagents used in the study were as follows: Folin–Ciocalteau's phenol reagent (2 N) purchased from Merck (Darmstadt, Germany), gallic acid, HCl, $FeCl_3 \cdot 6H_2O$ and sodium carbonate from Aldrich Chemistry (Munich, Germany). 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ, puriss,≥ 99.0%), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, a hydrophilic derivative of tocopherol, purum, ≥ 99%, for HPLC), were from Fluka (Buchs, Switzerland). Anton Paar alcolyzer plus, DMA 4500 density meter was used for monitoring of sugar and alcohol content in the samples, and UV/VIS spectrophotometer was used for determination of total polyphenol content.

Raspberry wine production

The raspberries pulp was prepared manually, and pulp without separation of juice, seeds and solid residue was fermented. The initial Brix value was 10.50 and the pH was 3.20. Eight kilograms of pulp was separated in the eight two liter glass bioreactors, and one kilogram of raspberry pulp was added to each of them. At 22°C four samples were fermented: one sample without sulfur dioxide and selected yeast, one sample with selected yeast and without sulfur dioxide, one sample without selected yeast and with sulfur dioxide and one sample with sulfur dioxide and selected yeast. The other four samples were the same as above, but these were fermented at 15°C. Where it was added, sulfur dioxide concentration was 75 mg/l free SO_2, and concentration of selected yeast was 0.4 g/L (dry weight). Fermentation was monitored by measurement of Brix values every 24 hours and it was considered complete when the Brix level was stable. Also, total polyphenol content in the pulp was monitored every 24 hours according to Folin Ciocalteau`s method. At the end of fermentation, seeds and pulp residue were separated from the wine by pressing and wine was transferred to bottles the capacity of 330 ml and stored at 5°C for sedimentation of the biomass. After 24 h, the beverages were transferred without aeration to new bottles and stored for further analysis.

Determination of total polyphenol content and antioxidant power

Total polyphenol content in the sample was determined every 24 hours from the beginning until the end of fermentation by Folin–Ciocalteau colorimetric method (Singleton et al., 1999). Twenty µl of the filtered and centrifuged sample were mixed with 1.58 ml distilled water, followed by addition of 100 µl Folin–Ciocalteau's phenol reagent. After eight min, 300 µl of 20% (w/v) sodium carbonate solution were added, and the mixture was incubated for two hours at 20°C. Absorbance of each sample in triplicate was measured at 765 nm using a spectrophotometer. Total polyphenol content was expressed as mg gallic acid equivalent per liter of sample (GAE). Ferric Reducing Antioxidant Power (FRAP) method (Benzie and Strain, 1996) was modified to a semi microscale. A portion of an aqueous 10 mmol/l solution of TPTZ reagent in 40 mmol/l HCl was mixed with the same volume of 20 mmol/l $FeCl_3 \cdot 6$ H2O and ten times higher volume of acetate buffer of pH 3.6 (3.1 g sodium acetate and 16

ml acetic acid per litre). The mixture was incubated at 37°C for five minutes. 100 µl diluted wine were mixed with 300 µl distilled water, followed by addition 3 ml of the Fe3+-TPTZ mixture and incubated for 40 min (after reaching plateau), and the absorbance at 593 nm was read. Trolox (1 mmol/l) was used for calibration. For statistical analysis, factorial ANOVA and Tukey`s HSD test were applied.

RESULTS AND DISCUSSION

The results of this study are shown in table 1 and 2. As seen in table 2, total polyphenol content (TPC) during fermentation increases, depending on conditions of fermentation. Samples MM1, MM2, MM3 and MM4 were fermented at 22°C, and TPC at the end of fermentation was higher than for samples MM5, MM6, MM7 and MM8 which were fermented at 15°C. In all samples, during the fermentation, TPC was constantly increasing, reaching its maximum values after 72 hours of fermentation. After 72 hours of fermentation TPC was decreasing in all of the samples until the end of the process. It may have been the result of irreversible precipitation caused by polyphenol compounds interactions with proteins in the medium. The highest TPC value was reached in MM3 sample which was fermented spontaneously at 22°C without selected yeast and with sulfur dioxide addition. The lowest TPC values were observed in samples were fermented with selected yeast and without sulfur dioxide. The obtained results indicate that temperature of fermentation and addition of sulfur dioxide have a strong influence on the TPC, while fermentation with selected yeast has the opposite influence. Relatively lower TPC value or samples fermented with selected yeast can be the consequence of absorption of polyphenol compounds on the cellular wall of yeast. At the end of fermentation, only in samples which are obtained after 120 hours of fermentation (final samples), Ferric Reducing Antioxidant Power (FRAP) was measured. Results of FRAP assay are shown in table 1. Results are expressed as mmol/l Trolox equivalents (mmol/l TE).

Table 1. The results of FRAP assay

Sample of wine	MM1	MM2	MM3	MM4	MM5	MM6	MM7	MM8
FRAP (mmol/l TE)	30.3	27.2	31.7	37.3	28.14	26.31	28.8	26

MM1-sample without selected yeast and sulfur dioxide at 22°C; MM2-sample with selected yeast and without sulfur dioxide at 22°C; MM3-sample without selected yeast and with sulfur dioxide at 22°C; MM4-sample with selected yeast and with sulfur dioxide at 22°C; MM5-sample without selected yeast and sulfur dioxide at 15°C; MM6-sample with selected yeast and without sulfur dioxide at 15°C; MM7-sample without selected yeast and with sulfur dioxide at 15°C; MM8-sample with selected yeast and with sulfur dioxide at 15°C

Table 2. Total polyphenol content of raspberry wine during fermentation

Sample of	**FC (mg GAE/L)**

wine	0 hour	24 hours	48 hours	72 hours	96 hours	120 hours
MM1	1504.3	1550.5	2337	2516.8	2012.5	2101.1
MM2	1504.3	1505	1977.5	2382	1809	2033.7
MM3	1504.3	1662.9	1670	2820	2112.3	2325.8
MM4	1504.3	1640.4	2045	2505.6	2090	2247.2
MM5	1504.3	1620	1814.5	1876.4	1865.2	1730.3
MM6	1504.3	1714.3	1796.8	1820.2	1629.2	1561.8
MM7	1504.3	1826.7	1870.1	1910.1	1775.3	1786.5
MM8	1504.3	1513.4	1694	1707.8	1640.4	1618

Table 3. Results of statistical analysis
*N.S. – statistically not significant difference; * - statistically significant difference; ** - statistically very significant difference*

	Sample of wine	MM1	MM2	MM3	MM4	MM5	MM6	MM7	MM8
Sample of wine	Value for TPC	2101.1	2033.7	2325.8	2247.2	1730.3	1561.8	1786.5	1640.4
MM1	2101.1	/	N.S.	N.S.	N.S.	*	**	*	*
MM2	2033.7		/	N.S.	N.S.	*	**	N.S.	**
MM3	2325.8			/	N.S.	**	**	**	**
MM4	2247.2				/	**	**	*	**
MM5	1730.3					/	N.S.	N.S.	N.S.
MM6	1561.8						/	N.S.	N.S.
MM7	1786.5							/	N.S.
MM8	1640.4								/

Figure 1 shows the results of Folin Ciocalteau assay using the box plot, and they are presented as the mean of three replication with standard deviation and standard error.

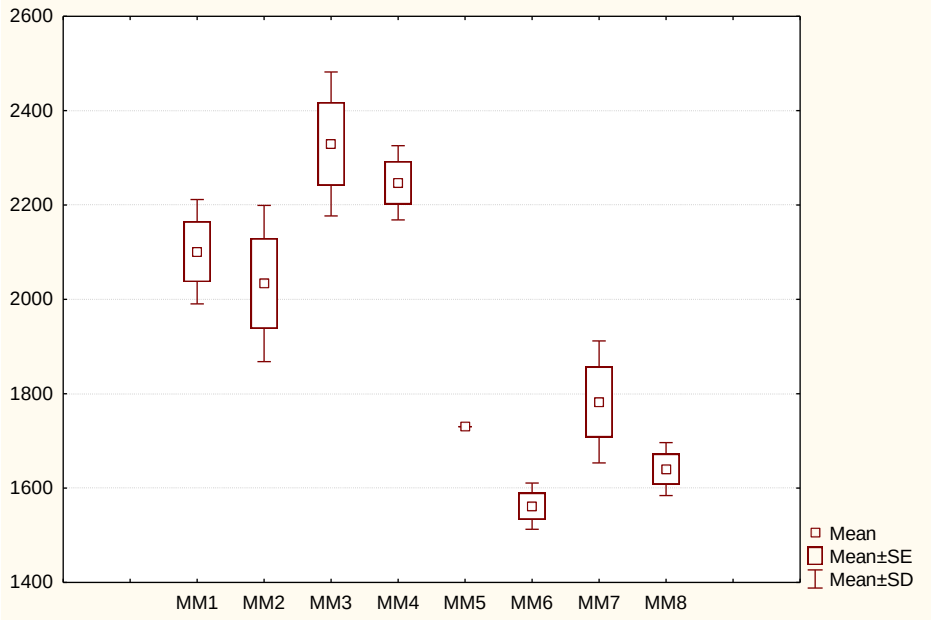

Figure 1. The results of Folin-Ciocalteau assay

The results of statistical analysis are shown in table 3. As shown, there are no statistically significant difference between TPC of samples which are fermented at the same temperature. This may be the results of two different phenomena: very strong influence of temperature on the TPC value and/or the fact that sulfur-dioxide and selected yeast have particularly no influence at all. On the other hand, TPC of samples which were fermented at different temperatures is in the most of cases statistically very significant. This fact supports the conclusion that temperature of fermentation has strong influence on the phenol compounds extraction during fermentation.

CONCLUSIONS

Results of this study show that conditions of fermentation of raspberry pulp have direct influence on the total polyphenol content in raspberry wine. Fermentation temperature strongly influence TPC content of wine. For example, value of TPC in the sample without selected yeast and with sulfur dioxide addition which fermented at 22°C was 2325.8 mg GAE/L, and for sample with selected yeast and without sulfur dioxide addition which fermented at 15°C was 1561.8 mg GAE/L. Statistically, in this study, sulfur-dioxide addition and selected yeast have no influence on the TPC. During fermentation, total polyphenol content in all of samples constantly increases to 72 hours of fermentation whereas reach its maximum.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III 46010 financed by the Ministry of Education and Science, Republic of Serbia.

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THE IMPACT OF *FUSARIUM GRAMINEARUM* CONTAMINATION LEVELS OF BARLEY ON DEOXYNIVALENOL AND ZEARALENONE CONTENT IN KILNED MALT

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ABSTRACT: The aim of this study was to establish the impact of initial *F. graminearum* contamination level of barley on deoxynivalenol (DON) and zearalenone (ZEA) content in kilned malt. The influence of applied unit operations during the malt production on development of the above-mentioned fungus and its ability to synthesize DON and ZEA was also considered. Barley samples contaminated with different initial *F. graminearum* contamination levels (0, 10 and 20%) were subjected to micromalting procedure. Barley samples, green malt and kilned malt underwent microbiological and toxicological analyses at which the share of *F. graminearum* contaminated grain and concentrations of mycotoxins were determined. The results show that the strongest proliferation of fungus occurred during steeping and germination, while the drying phase significantly reduced the contamination level. Furthermore, the results indicate that barley with higher initial *F. graminearum* contamination level ensured higher concentrations of mycotoxins in kilned malt. The highest DON concentrations were determined in germ/rootlets samples, while the highest ZEA concentrations were determined in kilned malt.
Key words: *barley, Fusarium graminearum, malting, deoxynivalenol, zearalenone*

INTRODUCTION

Barley is a wide spread commodity used for human consumption. It is used for malt production, as a basic element for beer production. Barley grains represent nutritionally rich substrate and they are therefore suitable for microbial growth and proliferation (Kocić-Tanackov et al., 2005).
The most interesting microorganisms that cause damage on the barley grain are fungi, first of all of genus *Fusarium*. These fungi are naturally present on the grain and they proliferate during the malting process depending on different factors: initial contamination of grain, interaction between species of microbial population, nutritive characteristics of the grain, and most of all, process parameters (temperature, aeration, additives, and humidity) (Noots et al., 1998). Fungi contaminate barley during flowering, in the field, but they can additionally spread during transport and storage. The results of *Fusarium* infection are yield decrease, lower average seed dimensions, decrease in nutritive value, loss of color and changes in smell and taste (Vanne & Haikara, 2001; Siranidou et al., 2001; Kocić-Tanackov et al., 2005). *Fusarium* head blight (FHB) is a severe disease of small grain (Perkowski et al., 2003), that can be partially controlled by crop rotation (highest incidence of *Fusarium* is when barley was grown after corn), tillage, choice of barley cultivar and application of fungicides. Humidity content (rain) during barley anthesis has a huge impact on FHB development (Váňová et al., 2004).
Another downside of *Fusarium* infection are mycotoxins which are accumulating in the grain. Mycotoxins are secondary fungal metabolites that contaminate food and feed and cause toxic effects in humans and animals (Váňová et al., 2004). Being the most prevalent of the *Fusarium* toxins, deoxynivalenol (DON, vomitoxin) is one of the most important indicators of

quality and safety of malting barley (Salas et al., 1999). DON is a potent protein synthesis inhibitor via binding to the ribosomes in eukaryotic cells. In animals, in low or moderate concentrations it can cause anorexia, and higher doses cause vomiting (Legzdina & Buerstmayr, 2004). Mycotoxins from raw material that is used for brewing, like barley or wheat, transfer into malt and from there they can transfer to the final product: beer, which can be detrimental for human health (Vaughan et al., 2005; Lancova et al., 2008). It has been reported that 80-93% of DON from malt ended up in beer (Schwarz et al., 1995). Similar research was conducted by Noots et al. (2003) in which was determined that 80 % of DON was synthesized after steeping.
Noxious effects of zearalenone (ZEA) on humans and animals, primarily reproductive and developmental disturbances (Zinedine et al., 2007), represent a great challenge for the food and feed industry. As a secondary metabolite of *Fusarium* species, ZEA is among the most frequently detected mycotoxins in cereals. Widespread contamination with major fungal producers of this mycotoxin (Krstanović et al., 2005) and its presence (contaminating up to 30% of total cereal production) have also been reported in Croatia (Šegvić Klarić et al., 2008).
This paper refers to how different degrees of *Fusarium* infection influence mycotoxin levels in starting barley, green malt and dry malt.

MATERIAL AND METHODS

The analyses were conducted on barley, artificially inoculated in the field by *Fusarium graminearum,* obtained by the Faculty of Agriculture in Osijek, and non infected barley obtained by the Agricultural Institute in Osijek. Starting contamination of contaminated barley was 34%. To gain three samples with diverse contamination levels (0%, 10% and 20%), starting barley sample was diluted in a way that contaminated and uncontaminated barley were mixed in different scales. Real *F. graminearum* contamination degree was determined in unmalted barley samples according to MEBAK (1997). Manitol agar with antibiotics and fungicides was prepared. To provide surface disinfection of grains, 50 g of barley was soaked in 1% NaOCl for 10 minutes and then rinsed with sterile water. Hundred grains were carefully sorted onto manitol agar in Petri dishes and incubated in dark place at 30 °C. This procedure was conducted in parallels and as a final result, mean value was taken into account. Prepared barley samples with diverse contamination levels were micromalted according to MEBAK procedure (MEBAK, 1997). Hulled barley samples were weighed (250 g) and put into eight malting dishes.
Micro-malting was performed according to MEBAK standard procedure (MEBAK, 1997) in a laboratory incubator (ClimaCell, MMM Medcenter Einrichtungen, München, Germany). The kilning of green malt was also performed according to the MEBAK protocol. After drying, malt was transferred into paper bags and kept at room temperature for three days for moisture equilibration. Real *F. graminearum* contamination degree in malt was determined also according to MEBAK (1997).

Toxicological analysis

Chemicals and apparatus

A Ridascreen® FAST DON fast and ZEA kit for ELISA, competitive enzyme immunoassays, were provided by R-Biopharm (Darmstadt, Germany). Each kit contained a microtiter plate with 96 wells coated with specific antibodies; standard solutions of DON (at concentrations of 0, 0.222, 0.666, 2 and 6 µg/mL) and ZEA (at concentrations of 0, 0.05, 0.15, 0.45, 1.35 and 4.05 ng/mL); peroxidase conjugated DON/ZEA; anti- DON/ZEA antibody; substrate/chromogen solution (urea peroxide/tetramethylbenzidine); stop solution and washing/dilution buffers. ELISA method was performed by ChemWell (Awareness Technology 2910, Inc., USA). DON and ZEA standards were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany).

Sample preparation and extraction procedure
Samples (barley, green malt, malt, germ/rootlets) were grounded on an analytical mill (Cylotec 1093, Tecator). After grinding, five grams of each sample was extracted with 100 mL of distilled water for DON analysis and with 25 mL of methanol/water (70/30) for ZEA analysis. The extraction was performed by vigorous shaking for three minutes on a shaker and the extracts were filtered through a filter paper (Whatman, Black Ribbon). The supernatants obtained were appropriately diluted and used for ELISA tests according to the manufacturer's instructions.
Analysis of DON and ZEA
DON: Fifty µL of enzyme conjugate and 50 µL of anti-DON antibody solution were added to 50 µL of standards and prepared samples. Microwell holder was mixed gently and incubated for 5 min at room temperature. After incubation, the wells were washed 3 times with 250 µL of distilled water. Then, 100 µL of substrate/chromogen solution was added to each well and incubated for 3 min at room temperature in the dark.
ZEA: Fifty µL of diluted enzyme conjugate was added to 50 µL of ZEA standards and prepared samples. Microwell holder was mixed gently and incubated for 2 h at room temperature. After incubation, the wells were washed 3 times with 250 µL of distilled water. Then, 50 µL of chromogen solution and 50 µL of substrate solution was added to each well and incubated for 30 min at room temperature.
After these steps, the reactions in both analyzes were stopped by adding 100 µL of stop solution and absorbance was measured at 450 nm. The concentrations of DON/ZEA in samples were calculated by mathematical interpolation from the calibration curve and multiplication with the corresponding dilution factor. Statistical data analysis was performed by use of the Statistica Ver. 6.1. software (StatSoftInc. 1984-2003, USA). All mycotoxin analysis were performed at the Laboratory for Analytical Chemistry, Croatian Veterinary Institute.

RESULTS AND DISCUSSION

Three samples of barley, contaminated with *F. graminearum*, were prepared for the experiment. Samples were contaminated with different degrees of contamination (0%, 10% and 20%). One batch underwent malting with water change during the steeping process and the other batch underwent malting without water change.
Table 1 shows results concerning predicted and actual contamination level determined after microbiological analysis of prepared samples. Actual contamination level was expressed as a number of contaminated kernels (cherry pigment) regarding total number of kernels on selective medium. As expected for this kind of sample, in respect to predicted contamination level, a slight deviation from actual contamination level can be noticed. Microbiological analysis of green malt showed (Table 1**)** that during steeping and germination an increase of microbiological contamination occurred, regarding starting sample. This is in accordance with previous researches (Flannigan et al., 1984; Haikara et al., 1977) which showed that during malting process the starting *F. graminearum* contamination level can increase from 15 – 90%. Cross contamination of kernels with fungi occurs during steeping, when through steeping water, due to release of conidia, mycelium and ascospores, contaminants cross over from contaminated to uncontaminated kernel (Schwartz et al. 1995). No significant difference was noticed between the batch with changed steeping water and the one that had no steeping water change. During kilning, a significant decrease in contamination of all samples occurred, regarding green malt samples and starting barley samples (Table 1). Kilning was carried out in temperatures ranging from 50 – 80 °C, and this lowers the kernel moisture below 5%. This results with decrease in *F. graminearum* contamination level in dry malt.

Table 1. Microbiological contamination of samples collected during the malting process

Batch	Barley		Green malt	Dry malt
	Predicted contamination level/%	Actual contamination level/%	Contamination level/%	
Steeping water change				
0	0	0	0	0
10	10	7	13.5	4
20	20	17.5	52	7
No steeping water change				
0	0	0	0	2
10	10	5.5	10.5	9.5
20	20	19	39.5	7.5

Table 2. Concentrations of DON in samples collected during the malting process

Batch	c(DON) / mg/kg			
	Barley	Green malt	Dry malt	
			Grain	Germ/rootlets
Steeping water change				
0	< 0.222	< 0.222	< 0.222	< 0.222
10	1.790	1.906	0.496	5.804
20	4.222	1.534	2.534	8.124
No steeping water change				
0	0.542	< 0.222	< 0. 222	< 0.222
10	2.082	0.950	0.842	3.716
20	2.476	2.338	2.566	6.544

Table 3. Concentrations of ZEA in samples collected during the malting process

Batch	c(ZEA) / μg/kg			
	Barley	Green malt	Dry malt	
			Grain	Germ/rootlets
Steeping water change				
0	< 0.222	8.789	3.147	20.034
10	1915.758	2099.999	760.934	53.177
20	2509.717	3207.851	3238.399	225.735
No steeping water change				
0	22.215	9.044	21.388	43.467
10	1914.758	2027.373	1310.728	83.174
20	1257.563	3002.181	2680.669	428.695

Tables 2 and 3 show contamination levels of barley, green malt, dry malt and germ/rootlets with mycotoxins DON and ZEA. The results of starting barley analysis indicate that increase of *F. graminearum* contamination level, increases the DON and ZEA concentrations. During the malting process an increase of DON and ZEA concentration occurred in all samples, in respect to starting barley sample.

Toxicological analysis of green malt also showed increase of DON and ZEA concentrations. The increase in DON concentrations can be explained by *de novo* synthesis of DON during the later germination stages. Some researchers have shown that DON concentrations decrease occurs during the steeping phase (Schwarz et al. 1995). This can be attributed to the fact that DON is water-soluble and it transfers into steeping water. Schwartz et al. (1995) have determined that after five-day germination, DON concentrations become significantly higher than the ones in steeped grains (18 – 114%). DON concentrations in both batches slightly decreased in dry malt, in respect to green malt (Table 2), and ZEA concentrations in

dry malt slightly increased in respect to green malt (Table 3). No significant difference has been observed between the batches.

Furthermore, the results indicate that mycotoxins were not equally distributed in dry malt since DON concentrations in germ/rootlets were significantly higher than in dry malt kernel (Table 3). The germ is the place of the most intense fungi growth, so the DON concentrations in germ are the highest. Thus, degermination procedure actually decreases the mycotoxicological malt contamination. ZEA concentrations in the germ were lower than DON. This can be attributed to chemical compounds found in germ. It is presumed that the sugars from the germ react with ZEA and form a "masked" conjugate ZEA-glycoside.
Due to mycotoxins transfer from starting barley to final product – beer, it is important to monitor mycotoxins and to suppress fungi growth during the malt and production processes.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project 113 - 1780691 - 0538 "Unapređenje zdravstvene sigurnosti u proizvodnji i preradbi žitarica", financed by the Ministry of Science, Education and Sports, Republic of Croatia.

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THE CHANGES OF THE AMOUNT OF AMINO ACIDS IN FERMENTED MILK ENRICHED WITH FLAKES FROM BIOLOGICALLY ACTIVATED HULL-LESS BARLEY GRAIN AND MALT EXTRACT

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ABSTRACT: The changes of the amount of amino acids in milk fermented with YF-L811 enriched with flakes from biologically activated hull-less barley grain in concentration of 5% and with malt extract in different concentrations (2%, 4% and 6%) were studied. Pasteurized milk, freeze-dried culture YF-L811 (Chr.Hansen, Denmark), flakes from biologically activated hull-less barley grain (Latvia) and malt extract (Ilgezeem, Latvia) were used for experiments. The fermentation process was realized at 43 ^{o}C for 4 hours. The amount of amino acids was determined by Method AS/HPLC-MS.
Results showed that the ability of yoghurt culture YF-L811 (containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) to produce the amino acids can be promoted by adding flakes from biologically activated hull-less barley grain and malt extract. The amount of amino acids in the fermented milk samples depends on the concentration of added malt extract. The highest content of amino acids was determined in fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 4%. It was established significant differences ($p<0.05$) among fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 4% (the total amino acid content – 11.899 mg·100 g^{-1}) and fermented milk samples with flakes from biologically activated hull-less barley grain and malt extract in concentration of 2% and 6% (the total amino acid content – 4.714 mg·100 g^{-1} and 6.609 mg·100 g^{-1}).
Key words: *amino acids, hull-less barley grain, malt extract, fermented milk*

INTRODUCTION

Dairy products are an important part of functional food. However there are possibilities to find new ways to enrich traditional milk products with bioactive compounds. One of these is to add grain, because it contains high amount of proteins, vitamins, dietary fibre, etc.
Barley is a widely-consumed cereal due to its dietary and technological properties, but mainly (80-90%) barley production is for animal feed and malt (Liu and Yao, 2007). There are growing interests in barley products due to its bioactive compounds, such as β-glucans, tocols (Bonoli et al., 2004; Sharma and Gujral, 2010) and due to its phenolic and amino phenolic compounds (Goupy et al., 1999). The results of research show that the nutritional value of hull-less barley grain can be further increased with biological activation. This includes synthesized vitamin C and increased content of dietary fibre, vitamins B_2, E and niacin during hull-less barley grain biological activation time (Rakcejeva, Skudra, 2007). Therefore biologically activated hull-less barley grain can be used as a good addition for the development of new products.
Barley and malt are now gaining renewed interests as ingredients for the production of functional foods due to their concentration of antioxidant compounds (Qingming et al., 2010). Qingming et al. (2010) have reported that malt contains many phenolic compounds and significant antioxidant activities *in vitro* and *in vivo*. Malt should be considered as a new source of natural antioxidant for dietary needs.
Therefore, both valuable products – biologically activated hull-less barley grain and malt extract were added to fermented milk in order to increase the nutritional value of the final products. The task of the research was to investigate the changes of the amount of amino

acids in fermented milk with YF-L811 enriched with flakes from biologically activated hull-less barley grain and malt extract.

MATERIAL AND METHODS

The research was performed at the microbiological laboratory of the Department of Food Technology of Latvia University of Agriculture and at the laboratory of biochemistry and microbiology of the Scientific Institute "Sigra" of the Latvia University of Agriculture.
Pasteurized milk with fat content 2.5% and the yoghurt culture YF-L811, containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Chr.Hansen, Denmark), were used for experiments. The culture was stored at -18 ^{o}C.
Flakes from biologically activated hull-less barley grain (Latvia) in concentration of 5% and malt extract (Igezeem, Latvia) in different concentrations (2%, 4% and 6%) were added to milk. The nutrition composition of flakes from biologically activated hull-less barley grain and of malt extract was as follows: protein 13.53 g·100 g^{-1} and 4.00 g·100 g^{-1}, fat 2.70 g·100 g^{-1} and 0, carbohydrate 1.84 g·100 g^{-1} and 73.00 g·100 g^{-1}. Milk samples with flakes from biologically activated hull-less barley grain and malt extract were inoculated with yoghurt culture and fermented at 43 ^{o}C for 4 hours. The control sample was prepared without the flakes from biologically activated hull-less barley grain and malt extract for comparing results.
The content of amino acids was determined by Method AS/HPLC-MS. The differences in the amount of amino acids between the samples were analyzed using the analysis of variance (ANOVA). T-test was applied to compare the mean values, and p-value at 0.05 was used to determine the significant differences.

RESULTS AND DISCUSSION

Lactic acid bacteria can synthesize amino acids; that is determined by the proteolytic character of lactic acid bacteria (Simova et al., 2006). As it is known, these qualities of micro-organisms are genetically determined. In the mixed yogurt culture, *Lactobacillus bulgaricus* has higher proteolytic activity than *Streptococcus thermophilus*, and it has been proven that the free amino acids produced by *L.bulgaricus* are used by *S.thermophilus* (Beshkova, 1998), because *S.thermophilus* has complex amino acid requirement and its growth is influenced by the availability of free amino acids from the growth medium (Guimont, 2002). The primary importance of all amino acids for the growth of *S.thermophilus* is glutamic acid (Neviani et al., 1995). This is one of the factors to shape mixed cultures. The total amino acids content in fermented milk reflects the balance between proteolysis and assimilation by means of bacteria (Wood, 1981).
The content of essential amino acids in fermented milk samples depending on the added flakes from biologically activated hull-less barley grain and malt extract concentration is presented in Table 1.
When evaluating the content of essential amino acids in fermented milk samples with flakes from biologically activated hull-less barley grain and malt extract, it is apparent that the added flakes from biologically activated hull-less barley grain and malt extract affected the amino acids content in the product. Comparing the total content of essential amino acids of control sample (0.409 mg·100g^{-1}) with milk (10.900 mg·100g^{-1}) (Beitane, 2005), it was shown that the obtained results of the experiments confirmed achievements of the research by Oberman and Libudzisz (1998) that lactic acid bacteria require a wide range of amino acids for growth. By adding the flakes from biologically activated hull-less barley grain and malt extract, the content of essential amino acids in fermented milk can be increased. The amount of amino acids in the fermented milk samples depends on the concentration of added malt extract. The highest content of amino acids was determined in fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 4% (1.508 mg·100 g^{-1}). It was established significant differences ($p<0.05$)

among fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 4% and fermented milk samples with flakes from biologically activated hull-less barley grain and malt extract in concentration of 2% and 6%, as well as control.

Table 1. The amount of essential amino acids in fermented milk samples with flakes from biologically-activated hull-less barley grain and malt extract, mg·100 g^{-1}

Amino acids	Control	Concentration of malt extract		
		2%	4%	6%
Threonine	0.002	0.002	0.016	0.003
Valine	0	0.001	0.004	0.001
Methionine	0	0	0	0
Isoleucin	0.002	0.001	0.011	0.001
Leucine	0.049	0.088	0.134	0.143
Phenylalanine	0.004	0.006	0.011	0.006
Tyrosine	0.001	0.001	0.007	0.001
Lysine	0.351	0.342	1.325	0.540
Total	**0.409**	**0.441**	**1.508**	**0.695**

Results are given as mean values

Comparing the content of essential amino acids in fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 4% and control, it appears that the presence of flakes from biologically activated hull-less barley grain and malt extract influence the content of all essential amino acids except methionine.
Similar tendencies can be observed with the content of non-essential amino acids in fermented milk samples (Table 2).

Table 2. The amount of non-essential amino acids in fermented milk samples with flakes from biologically activated hull-less barley grain and malt extract, mg·100 g^{-1}

Amino acids	Control	Concentration of malt extract		
		2%	4%	6%
Aspartic acid	0.007	0.007	0.037	0.012
Serine	0.007	0.001	0.023	0.002
Glutamic acid	0.009	0.013	0.098	0.022
Proline	0.001	0	0.002	0.001
Glycine	0.004	0.001	0.025	0.002
Alanine	0.002	0.001	0.002	0.001
Histidine	3.451	3.494	7.359	4.757
Arginine	0.751	0.756	2.811	1.117
Cystine	0	0	0.034	0
Total	**4.232**	**4.273**	**10.391**	**5.914**

Results are given as mean values

Evaluating the data of Table 2, it was obvious that the highest content of non-essential amino acids had fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 4% (10.391 mg·100 g^{-1}). It was established that the content of all non-essential amino acids was higher except alanine in comparing with control.
The results of statistical analysis have shown that there were significant differences ($p<0.05$) between fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 4% and fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 2%, as well as control.
Summarizing the obtained results of the content of amino acids in fermented milk samples with flakes from biologically activated hull-less barley grain and malt extract, it could be concluded that one of the possible ways to promote the ability of yoghurt culture YF-L811 to

synthesize the amino acids in milk in order to elevate the nutritional value of final product, is by the addition of flakes from biologically activated hull-less barley grain and malt extract.

CONCLUSIONS

The ability of yoghurt culture YF-L811 to produce amino acids can be promoted by adding flakes from biologically activated hull-less barley grain and malt extract. The amount of amino acids in the fermented milk samples depends on the concentration of added malt extract. The highest content of amino acids was determined in fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 4%. It was established significant differences ($p<0.05$) among fermented milk sample with flakes from biologically activated hull-less barley grain and malt extract in concentration of 4% and fermented milk samples with flakes from biologically activated hull-less barley grain and malt extract in concentration of 2% and 6%.

ACKNOWLEDGEMENTS

This paper is a result of the research within the State Research Programme “Sustainable use of local resources (earth, food, and transport) – new products and technologies (NatRes)” (2010-2013) Project No. 3. „Sustainable use of local agricultural resources for development of high nutritive value food products (Food)”.

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THE EFFICIENCY OF *IN VITRO* ADSORPTION OF MYCOTOXINS BY ADSORBENTS OF PLANT ORIGIN

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ABSTRACT: The paper describes i*n vitro* model for evaluation of ability of two adsorbents of plant origin, to adsorb different mycotoxins. Fresh harvested tissues of submerged aquatic plant *Myriophyllum spicatum*, obtained from Sava lake – Belgrade, was washed with HCl solution (3%) and then with distilled water. Plant material was afterwards dried for few days at room temperature, then for 6 hours at 50ºC, crushed and sifted. Peach stones, obtained from "Vino Župa" - Aleksandrovac, were separated from soft fruit residues, washed, dried at room temperature, crushed and separated from kernels. The peach stones were further used as milled shell fractions.
A total of six mycotoxins: aflatoxin B1 (AFL), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZON), diacetoxyscirpenol (DAS) and T-2 toxin were tested *in vitro*. For adsorption experiments crude extracts of mycotoxins were used. The ability for binding mycotoxins was evaluated in the electrolyte 0.1 M K_2HPO_4 (pH 3.0 and 6.9, respectively). Mass ratio of individual mycotoxin and natural mineral adsorbent was 1:5000. The experimental mixtures were incubated for 1 hour on a rotary shaker (185 rpm) at room temperature (22-25^{o}C). The extractions of nonadsorbed mycotoxins from the filtrates were performed with organic solvents, and their quantifications were done by thin-layer chromatography.
M. spicatum bound more (94.7-96.0%) of applied AFL B1 than peach stones (73.3-80.0%). In the case of OTA higher adsorption indexes were observed by peach stones (50.0-66.7%). Binding of DON has been observed only by peach stones. Its adsorption index varied from 25.0 to 50.0% depending on the pH of the used electrolyte. *M. spicatum* adsorbed more ZON at pH 6.9 while peach stones adsorbed more ZON at pH 3.0. In the case of type A trichothecenes (DAS and T-2 toxin) adsorbents of plant origin bound only T-2 toxin. The amount of adsorbed T-2 toxin ranged from 16.7 (pH 3.0) to 33.33% (pH 6.9).
Key words: *mycotoxins, adsorbents of plant origin, in vitro*

INTRODUCTION

Biosorbtion technique for treatment of industrial waste waters by living or non-living biomass has been proven to be an excellent method for removal of toxic contaminants that offers significant advantages: low cost, availability, profitability, easy operating and efficiency (Dhir and Kumar, 2010).
Myriophyllum spicatum (Eurasian water milfoil) is a submerged aquatic plant, native to Europe, Asia, and North Africa, and it was introduced into North America where is invasive species (Couch and Nelson, 1985). *M. spicatum* reproduces primarily by vegetative fragmentation and it can quickly recolonize areas that have been cleared of the species, because of the viability of even small fragments (Aiken et al., 1979). The control methods for growth of *M. spicatum* can be classified as physical (**mechanical harvesters and chopping machines)**, chemical, and biological.
Activated carbon (AC) prepared by pyrolysis of carbonaceous organic compounds (mostly nutshells, wood, paddy husk, etc.) is a general adsorbtive material with a large surface area and excellent adsorbtive capacity. It has been recommended as a general toxin adsorbing agent and is routinely recommended for various digestive toxicities. It is well known that AC

can be used as one of the very effective, non toxic mycotoxin-adsorbing agent (Whitlow, 2006). In order to decrease the costs of adsorbent preparation, which is in preparing of AC inevitable, the authors have tried to explore the possibility of application of raw, untreated lignocelullosic material for adsorption of different types of mycotoxins.
The aim of the presented study was to evaluate *in vitro* the ability of two adsorbents of plant origin, to adsorb six different mycotoxins: aflatoxin B1 (AFL), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZON), diacetoxyscirpenol (DAS) and T-2 toxin.

MATERIAL AND METHODS

Preparation of adsorbents of plant origin

Samples of fresh harvested tissues of *M. spicatum* (**MS**), used for experiment, were taken from Sava Lake. Plant biomass was washed with diluted HCl solution (3%) and then with distilled water three times before drying. Washed plant material was exposed to air and dried for couple days at room temperature and then dried at 50 ºC for 6 hours, crushed and sifted to a particle size less then 100 µm.
Peach stones (**PS**) used as another raw material for adsorption of mycotoxins was obtained from "Vino Župa" Company from Aleksandrovac, where the stones have been disposed as by-product waste from their Juice Factory. After sampling, the fruit stones were separated from soft fruit residues, washed, dried at room temperature, and kept in polypropylene bottles for further treatment. Prior to experiment, all the samples were crushed and separated from kernels, which were discharged, so for further analysis only hard stone part was taken. The peach stones were further used as milled shell fractions with diameter less than 100 µm.
Both adsorbents of plant origin were prepared in the Institute for Technology of Nuclear and Other Mineral Raw Materials, Belgrade.

Production, quantification and isolation of mycotoxins

Aflatoxin B1 (AFL), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZON) were produced employing solid substrate fermentation per the methods of Bočarov-Stančić et al. (2009a), Bočarov-Stančić et al. (2010) and Bočarov-Stančić et al. (2009b), respectively. Type A trichothecenes (diacetoxyscirpenol - DAS and T-2 toxin) were biosynthesized by submerged fermentation in liquid medium (Bočarov-Stančić et al., 2007). The respective fungal cultures used were: *Aspergillus flavus* GD-2 (leg. prof. dr G. Dimić, Technological Faculty, Novi Sad, Serbia), *A. ochraceus* CBS 108.08, *Fusarium graminearum* GZ-LES (leg. dr J. Lević, Maize Research Institute, Belgrade-Zemun, Serbia), *F. graminearum* D2 (leg. dr A. Bočarov-Stančić, Bio-Ecological Centre, Zrenjanin, Serbia), *F. semitectum* SL-B (leg. dr A. Bočarov-Stančić, Bio-Ecological Centre, Zrenjanin, Serbia), and *F. sporotrichioides* ITM-391 (leg. dr A. Bottalico, Consiglio Nazionalle delle Richerce, Istituto Tossine a Micotossine da Parassiti Vegetali, Bari, Italy).
Isolations of mycotoxins and determinations of single mycotoxin content in solid substrates were done according to standard thin-layer chromatographic method for fodder analysis (The Official Gazette of SFRY, issue 15/87). Isolations of type A trichothecenes were done by ethyl acetate and their quantities were determined by thin-layer chromatographic (TLC) method according to Rukmini and Bhat (1978). Isolated crude toxins were evaporated to dryness and dissolved in following solvents: ethanol (AFL, OTA, ZON), ethyl acetate (DAS, T-2) and methanol (DON). The final concentrations of stock mycotoxins' solutions were 0.1 µg/µl (AFL) and 1 µg/µl (OTA, DON, ZON, DAS and T-2), respectively.

Experimental procedure

For adsorbtion experiments stock solution of AFL was diluted to 0.2 µg/ml, of ZON to 0.8 µg/ml, and of all other mycotoxins to 2.0 µg/ml with electrolyte (0.1M K_2HPO_4). pH value of electrolyte was adjusted with 0.1M HCl or 0.1 NaOH to 3.0 and 6.9, respectively.

The *in vitro* binding ability of *M. spicatum* and peach stones was tested as follows: aliquots (50 ml) of test solutions (containing 10 µg of AFL, 40 µg of ZON and 100 µg of all other tested mycotoxins) were added to Erlenmayer flasks (250 ml) containing 500 mg of single adsorbent in the case of OTA, DON, DAS and T-2 toxin, 200 mg in the case of ZON, and 50 mg in the case of AFL. Controls were prepared by adding of 50 ml of the test solutions without mineral adsorbent. The flasks were stoppered, incubated for 1 hour on rotary shaker (185 rpm) at room temperature (22-25 ^oC) and then filtered. Mycotoxins' concentrations in 25 ml aliquots of electrolyte with adsorbent (C) and without it (C_o) were determined, after extraction with 2 x 15 ml of organic solvents: benzene (ZON), benzene-acetonitrile (AFL), and ethyl acetate (OTA, DON, DAS and T-2) respectively, by TLC methods (The Official Gazette of SFRY, issue 15/87; Rukmini and Bhat, 1978). All analysis were performed in three replications.

The adsorption index of individual mycotoxin in percentages was calculated by the following formula:

$$Adsorption\ index = [\frac{C_0 - C}{C_0}]\ x\ 100$$

RESULTS AND DISCUSSION

Table 1. Chemical composition of adsorbents of plant origin, %

Parameter (%)	*Myriophyllum spicatum* (**MS**)	Peach stones (**PS**)
Dry matter	91.11	92.23
Moisture	8.89	7.77
Crude protein	17.95	1.26
Crude fat	1.28	0.05
Crude cellulose	23.33	58.05
Ash	17.64	0.42
Nitrogen free extracts (NFE)	30.91	32.45
Neutral detergent fiber (NDF)	33.38	71.12
Acid detergent fiber (ADF)	30.96	66.12
Lignin	6.33	16.54

The investigated adsorbents had very different chemical composition (Table 1). In relation to the peach stones *Myriophyllum spicatum* had a significantly higher content of protein, crude fat and ash, while the crude fiber content and lignocellulosic fractions (ADF, NDF and lignin) was noticeably smaller. Chemical composition of *M. spicatum*, (with the exception of ash content) is very similar with composition of some plants of the family *Fabaceae* (*Medicago sativa* and *Trifolium repens*). On the other hand, chemical composition of peach stones is similar or approximate to the composition of the peanuts husks or sunflower husks. Such a chemical composition of the studied mycotoxins adsorbents of plant origin indicates, among others, that they can be used as a feed additive (*M. spicatum*) or energetic material (peach stones) or even as carriers of certain active substances used in agriculture and industry.

Table 2. Adsorption indexes of tested adsorbents of plant origin at different pH values

Adsorbent	pH	Adsorption index (%)					
		AFL	OTA	DON	ZON	DAS	T-2
MS	*3.0*	*96.00*	*50.00*	*0*	*70.00*	*0*	*16.67*
	6.9	94.70	30.00	0	75.00	0	33.33
PS	*3.0*	*80.00*	*66.67*	*25.00*	*53.30*	*0*	*16.67*
	6.9	73.30	*50.00*	50.0	33.33	0	33.33

Aflatoxin B1. By the use of TLC method it was noted that M. spicatum binded more (94.7-96.0%) of applicated AFL than peach stones (73.3-80.0%) (Table 2). Influence of pH value of applied electrolyte on the binding of this mycotoxin was not significant. Obtained results are similar with the results of our previous investigations with natural mineral adsorbents (bentonite – BEN, diatomite – DIA and zeolite – ZEO). We have found that these minerals binded more than 95% of applicated AFL (Bočarov-Stančić et al., 2011).

Ochratoxin A. In the case of OTA higher adsorption indexes (50.0 - 66.67%) were observed by peach stones (Table 2). In both cases adsorbents of plant origin binded more OTA at pH 3.0 than at pH 6.9 as same as the most of mineral adsorbents (Thimm et al., 2001).

Deoxynivalenol. Adsorption of this type B trichothecene (DON) has been observed only by peach stones. Its adsorption indexes varied from 25.0 to 50.0% depending on the pH of the used electrolyte. Contrary to our previous results obtained in vitro with natural mineral adsorbents (Bočarov-Stančić et al., 2011) adsorption of this fusariotoxin was more pronaunced at neutral pH (6.9).

Zearalenone. Effect of electrolyte pH value on the binding of ZON was different. M. spicatum adsorbed more ZON at pH 6.9 while peach stones adsorbed more ZON at pH 3.0 (Table 2). Observed adsorption indexes for MS (70.0 - 75.0) and PS (33.33 - 53.3) were significantly higher then adsorption indexes achieved in vitro with natural mineral adsorbents (BEN, DIA and ZEO), when the largest binded quantity of ZON (12.2 to 37.0 %) was observed by ZEO (Bočarov-Stančić et al., 2011).

Type A trichothecenes. In the case of these fusariotoxins (DAS and T-2 toxin) adsorbents of plant origin (MS and PS) binded only T-2 toxin. The amount of binded T-2 toxin was the same in both cases and ranged from 16.67 (pH 3.0) to 33.33% (pH 6.9). That there is little or no beneficial effect against this type A trichothecene also showed the results of other authors obtained with different mineral adsorbents (Devegowda and Aravind, 2002; Bočarov-Stančić et al., 2011).

Adsorbents of plant origin tested in the present investigation are known as biosorbents of different toxic substances, but not mycotoxins. M. spicatum is capable of regulating salt intake independent of concentration in the aquatic environment (Anderson et al., 1966) and can be successfully used for the removal of heavy metals - lead, zinc and copper (Keskinkan et al., 2003 and 2007). On the other hand peach stones, produced from food industries as solid waste, can also be used for binding of lead ions from polluted water (Rashed, 2006) or aqueuos ammonia (Sotto-Garrido et al., 2003) and some textile dyes (Gercel et al., 2009). It is not surprising that peach stones was better biosorbent of mycotoxins in vitro conditions because it had much higher cellulose content then M. spicatum (Table 1). Although raw, untreated material was used in the present experiments it showed similar adsorption capacities as activated carbon, especially in the case of deoxynivalenol (Döll et al., 2004).

CONCLUSION

Both tested adsorbents of plant origin showed the highest adsorption indexes *in vitro* for aflatoxin B1 (73.3-96.0%).

Rather high adsorption indexes were obtained for zearalenone (33.3-75.0%), and ochratoxin A at pH 3.0 (50% - MS, and 66.67 – PS).

Deoxynivalenol bound only by peach stones (25% and 50%, respectively).
None of the investigated adsorbents adsorbed diacetoxyscirpenol.
The amount of bound T-2 toxin was rather small, although it was the same for MS, and PS (16.67 to 33.33%).
In tested laboratory conditiond the better adsorbent of plant origin was shown to be peach stones because it binded in vitro five (AFL, OTA, DON, ZON and T-2) out of six tested mycotoxins.
Investigated adsorbents, such as they are, can be recommended for the use in ruminants nutrition because ruminants are more tolerant then nonruminants on the presence of higher concentrations of mycotoxins in feed.
Nonetheless in vivo experiments are indispensable to proof the efficacy of investigated adsorbents (MS, and PS) and other ones of plant origin.

ACKNOWLEDGEMENTS

This paper is a result of the researches within the project TR 31023 and TR 31003 financed by the Ministry of Education and Science of the Republic of Serbia.

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THE INFLUENCE OF RAW MATERIALS AND FERMENTATION CONDITIONS ON THE POLYPHENOL CONTENT OF GRAPE BEER

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ABSTRACT: Over the last decades, there has been an increased interest in studying the potential health benefits of moderate wine and beer consumption. Studies indicate that wine and beer consumed moderately can have a very favorable effect on the overall health condition. The effect can be explained by a high content of antioxidants and other biologically active compounds, particularly polyphenols, which can prevent the occurrence of some diseases, especially cardiovascular disease. In this research, a special type of beer, the grape beer, was produced using three different grape varieties Prokupac, Pinot Noir and Cabernet Sauvignon. Beer samples were fermented using conventional brewer's wort and 20 % w/w and 30 % w/w of crushed grape. The fermentation was carried out using two different yeasts: *Saccharomyces pastorianus* industrial strain obtained from one of the Serbian breweries and wine yeast *Saccharomyces cerevisiae* K1-V1116. The influence of grape variety, the wort-to-grape ratio and yeast strains on the polyphenol content was examined. The total polyphenol content was determined according to the official EBC (European Brewery Convention) spectrophotometric method. The results suggested that grape varieties, their contents, as well as yeast strains had a very significant influence on the total polyphenol content of the obtained beer. The polyphenol content rang in samples went from 95.94 mg/L in beer without grape fermented by brewing yeast and up to 754.40 mg/L in beer with 30 % of Cabernet Sauvignon grape fermented by wine yeast.
Key words: *beer, grape, polyphenol, fermentation*

INTRODUCTION

Over the last decades, numerous studies have dealt with the possibility that certain foods can promote health and improve the prevention of many diseases. The term „functional food" was first introduced in Japan in the mid 1980s and refers to any processed food or food ingredient that may provide additional physiological benefit, such as preventing or delaying onset of various diseases, as well as meeting basic nutritional requirements (Dey et al., 2009). New types of food products with improved healthy function are a growing trend. The utilization of dietary compounds and natural products as potential disease prevention agents in the form of functional foods has become an important task in current health studies (Gerhauser, 2005). Numbers of research works have investigated the possible health benefits of moderate consumption of various alcoholic beverages, such as wine, beer, spirits, liqueurs etc (Corrao et. al., 1999). Many of these studies supports the hypothesis that moderate drinking of any alcoholic beverage, particularly red wine and beer, significantly reduces the risk of cardiovascular diseases (Corrao et. al., 1999). The protective effects of beer and red wine can be explained by a high content of natural antioxidants, particularly phenolic compounds. Antioxidants are "any substance that, when presents at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell, 2007). Among antioxidants, phenolic compounds are by far the most abundant in the diet. Many of these natural antioxidants, especially flavonoids and stilbenes, exhibit a number of bioactive effects, such as anti-inflammatory, antimicrobial, antiallergic, antithrombotic, anticancerogenic, antimutagenic, antiaging and vasodilatory activities (Jaganath and Crozier, 2010). Dietary antioxidants are able to scavenge of free radicals, thus preventing a number of degenerative diseases caused by oxidative stress (Heim et al., 2002). Beer and wine contain high amounts of

phenolic compounds and other valuable nutrients and can be an interesting basis for development of new functional products.

Beer is a worldwide low-alcohol beverage made from natural ingredients: barley (malt), water, hop and yeast. It has a very complex chemical composition (several hundred compounds have been so far characterized in beer) and may contain significant amounts of nutritional as well as non-nutritional components including carbohydrates, proteins, vitamins, amino acids, minerals and many others phytochemicals. Beer is a rich source of proteins, B type vitamins (B6, B12 and B9), minerals and soluble fibers (a liter of beer contains 20-60 % of recommended daily intake of fiber) (Gromes et al., 2000). In addition, beer is a rich source of bioavailable silicon (Powell et al., 2005). The antioxidant capacity of beer depends on the content of polyphenols and Maillard compounds. Beer polyphenols come mainly from barley (malt) (70-80 %) and hops (20-30 %), which are basic raw materials for its production. However, besides the influence of raw materials, the total antioxidant content of beer significantly depends on the brewing process used. Antioxidants have a very important role in malting and brewing due to their ability to delay and prevent oxidation reactions. Also, various antioxidants (sulfites, ascorbic acid etc.) can be added during the brewing process to improve flavor stability of products (Zhao et al., 2008). However, minimizing the use of additives and increasing the content of antioxidants from natural sources to improve flavor stability and increase the shelf-life of products are growing trend in food and beverage industry.

Wine is probably one of the oldest alcoholic beverages as confirmed by archeological record dating back more than 7.5 thousand years. It has most likely inspired more research and publications than any other beverage or food (Jackson, 2008). The effect of wine consumption on health has been intensively studied over the last several decades. Most studies confirmed that moderate drinking of red wine reduces the risk of cardiovascular diseases and can improve overall health. Such a red wine effect has usually been linked to its high content of polyphenols (Scalbert et al., 2005). The content of phenolic compounds in wines is conditioned by the grape variety and by other factors that influence the grape ripening, such as soil, geographical region and weather conditions. On the other hand, phenolic composition of wines largely depends on the winemaking process, which has the most important role in the extraction process of polyphenols from grapes (Paixao et al., 2007). In addition to their function of antioxidants, phenolic compounds can affect to wine sensorial properties, such as appearance, taste, mouth-feel, fragrance, astringency and bitterness.

Phenolic compounds of wine and beer are quantitatively and qualitatively different because the raw materials used in their production are completely different. Some classes of polyphenols, such as flavan-3-ols and flavonoles are present in similar amounts in both beverages. However, there are phenolic compounds mainly present in red wines (stilbenes and proanthocyanidins), or exclusively in beer (flavanones and chalcones) (Iacomino et al., 2009). The total amount of polyphenols in wine is several times (mainly 2-3) higher than in beer, but absorption of these natural antioxidants by an organism is as important as their presence in beverages. It has been shown that by consuming equal amounts of alcohol from beer and wine, blood contains approximately equal amounts of antioxidants (Baxter and Hughes, 2001). In a previous work, we investigated the possibility of producing a special type of beer by fermenting wort with different proportion of grape must (Veljovic et al., 2010). The obtained results indicated that special grape beer has unique sensorial profile completely acceptable for consumers and significantly higher content of polyphenols. Also, there are some beers on the world market that are produced with the addition of grape such as Vigneronne Cantillon.

The main objective of this study was to investigate the influence of grape varieties, proportions of grape in fermenting medium, yeast strains and conditions of fermentation on total polyphenol content of grape beers.

MATERIAL AND METHODS

Three different grape varieties were used: Prokupac, Pinot noir and Cabernet sauvignon. Prokupac is Serbian autochthonous variety, used for making table and top quality rose and red wines, while Pinot noir and Cabernet sauvignon are one of the best known red grape varieties used for producing top quality wines. Grapes were obtained from experimental school estate "Radmilovac" of Faculty of Agriculture, Belgrade. The wort and a bottom-fermenting industrial yeast strain *Saccharomyces pastorianus* used in this study were obtained from a local brewery. Wine yeast *Saccharomyces cerevisiae* K1-V1116 was purchased from Lallemand (Montréal, Canada). Sodium carboxymethyl cellulose (CMC), ethylenediaminetetraacetic acid (EDTA) and ferric ammonium citrate were purchased from Sigma-Aldrich (Steinheim, Germany). Concentrated ammonia was obtained from Centrohem (Belgrade, Serbia).

Fermentation

Each of the three grape varieties was used to prepare two different media for fermentation, one with 20 % and another with 30 % of grape mash. The grapes were sorted manually, washed in cold water to remove impurities, and the clean grapes were destemmed and crushed by hand. The wort and grape mash were mixed in different proportions (70:30 and 80:20) and the pH of obtained mixtures was adjusted to 5.3 with diluted solution of ammonium hydroxide. The fermentation mediums (4 liter) were poured into 5 L laboratory stainless steel fermenters and seeded with 80 mL of a yeast suspension, corresponding to 10-15 million yeast cells per milliliter of wort. The fermentation process was conducted according to the yeast strain used. Pitching was performed at 12°C or 15°C with *S. uvarum* and *S. cerevisiae* K1-V1116, respectively. When the real extract was decreased to 5 % w/w, the fermenting mixture was sent to a laboratory wine press. After pressing, liquid was transferred into an aging stainless steel vessel equipped with pressure control system. At the end of secondary fermentation, beer was filtered and bottled. Control beer samples were produced in the same way but without addition of grape mash.

Determination of total polyphenols

The total polyphenol content was determined according to the official EBC (European Brewery Convention) spectrophotometric method. Every beer sample (10 mL) was pipetted into a 25 mL volumetric flask where was mixed with 8 mL of CMC/EDTA reagent (10 g/L CMC containing 2 g/L EDTA). Afterwards, 0.5 mL of green ferric ammonium citrate solution (3.5 % w/v) and 0.5 mL of ammonia reagent (concentrated ammonia : water = 1:2) was added to the mixture and vigorously shaken. The total volume was adjusted to 25 mL with distilled water. The absorbance of red-colored solution was measured at 600 nm after 10 minutes. In blank sample, ferric reagent was replaced with distilled water. The content of polyphenols was calculated using the following formula:

$$\text{Polyphenol content (mg/L)} = (A_s - A_b) \times 820 \times F,$$

where A_s, A_b and F are the absorbance of the sample, absorbance of the blank and dilution factor, respectively.

Statistical analysis

All measurements were conducted in triplicate. Analysis of variance and significant differences among means were tested using ANOVA and Tuckey's HSD test.

RESULTS AND DISCUSSION

In our previous research, we found that it is possible to produce a very interesting and pleasant special type of beer made by fermenting mixture of wort and grape must, which was characterized with higher polyphenol content compared with commercial light beer (Veljovic et al., 2010). This is a continuation of the aforementioned research in which the

main aim was to investigate the influence of grape varieties and its proportions in fermenting medium, yeast strains and conditions of fermentation on total polyphenol content of grape beers.

The polyphenol content of analyzed beer samples with basic statistical parameters are shown in table 1. Variations of values for polyphenol content within individual samples were very small. The control beer fermented by *S. pastorianus* had the lowest content of polyphenols, while the highest values recorded in beer with 30 % of Cabernet sauvignon fermented by *S. cerevisiae*.

Table 1. Basic statistical parameters for polyphenol content of analyzed beer samples

Yeast	Samples	Mean (mg/L)	Min.	Max.	Variance	Std.dev.	Coef.var.	Std.err.
S. pastorianus	Cb	95.94	95.08	96.62	0.62	0.79	0.82	0.45
	PN20	154.16	153.73	154.99	0.52	0.72	0.47	0.42
	PN30	248.46	248.01	249.23	0.45	0.67	0.27	0.39
	P20	151.70	151.39	151.92	0.08	0.28	0.18	0.16
	P30	174.66	174.18	175.08	0.21	0.45	0.26	0.26
	CS20	257.48	256.98	258.13	0.35	0.59	0.23	0.34
	CS30	347.68	347.26	348.15	0.20	0.45	0.13	0.26
S. cerevisiae	Cb	102.50	102.33	102.65	0.03	0.16	0.16	0.09
	PN20	347.68	347.31	347.95	0.11	0.33	0.10	0.19
	PN30	380.48	379.89	381.05	0.34	0.58	0.15	0.34
	P20	332.10	331.72	332.76	0.33	0.57	0.17	0.33
	P30	371.24	371.00	371.69	0.15	0.39	0.11	0.23
	CS20	674.04	673.57	674.59	0.26	0.51	0.08	0.30
	CS30	754.40	754.12	754.86	0.16	0.40	0.05	0.23

Cb - control beer; PN20, PN30 - beers with 20 and 30 % of Pinot noir, respectively; P20, P30 - beers with 20 and 30 % of Prokupac, respectively; CS20, CS30 - beers with 20 and 30 % of Cabernet sauvignon, respectively; Std.Dev. - standard deviation; Coef.var. - coefficient of variation; Std.err. - standard error

Table 2. The results of analysis of variance

Factors	F	p
Grape variety	541487.7	0.000
Proportion of grape	1389661.3	0.000
Yeast strain	1411092.0	0.000
Grape variety*proportion of grape	139332.5	0.000
Grape variety*yeast strain	132800.8	0.000
Proportion of grape*yeast strain	327682.9	0.000
Grape variety*proportion of grape*yeast strain	35278.3	0.000

F - sample values applied test; p - level of significance (p≤0.05, difference is significant)

The results of the ANOVA test are shown in table 2. ANOVA indicates that all observed factors (grape variety, proportion of grape in fermenting medium and yeast strain) influence very significantly on the polyphenol content of samples. The combined effects of factors on the measured values were also very significant, which indicates that the impact of one factor depends on the level of the two other factors. All samples fermented by *S. cerevisiae* had higher amount of polyphenols compared with the appropriate samples fermented by *S. pastorianus*; in some cases, difference between these samples was greater than twice. One of the reason for better extraction of phenolic compounds during fermentation with yeast

strain *S. cerevisiae* K1-V1116 can be slightly higher temperature (15°) in comparison to fermentation with *S. pastorianus* (12°C). The beers produced with Cabernet sauvignon had the highest polyphenol content, while the lowest content was present in the beers with Prokupac. With increasing proportion of grapes in fermenting medium from 20 to 30 %, polyphenol content of obtained beers was also very significantly increased.
In addition, the obtained grape beers were characterized by a pleasant flower-fruit fragrance, enjoyable aroma and high fullness of taste, with prominent freshness and good after-taste.

CONCLUSIONS

The results obtained suggest that all examined grape beers had a higher amount of polyphenolic compounds compared to control beers. The grape variety, proportion of grape in fermenting medium and yeast strain had a significant influence on polyphenols content of grape beer. However, the interactions between factors were also significant, which indicates that these factors did not affect independently, but the impact of any factor depends on the level of the two other factors. The highest polyphenol content had the grape beer produced with 30 % of Cabernet sauvignon and fermented by *S. cerevisiae* K1-V1116, while the lowest value had the control beer fermented by *S. pastorianus*. These results are encouraging for further research and indicate the possibility of obtaining a wide range of special beers with increased polyphenol content.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education and Science, grant No. 46001.

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WHITE BRINED CHEESE AS A DELIVERY MEDIUM FOR PROBIOTIC BACTERIA

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ABSTRACT: The traditional white brined cow cheese was tested as a delivery medium for probiotic bacteria. The white brined cheese was manufactured by the traditional method. The cheese produced in this way has relatively short period of ripening, large percentage of fat and suitable pH values and therefore provides good conditions for survival of the probiotic culture. Two different initial concentrations of the probiotic bacterium, *Lactobacillus casei*, were used in this study. The traditional and probiotic type of cheeses were produced in a dairy plant in three separate batches. Due to the mild production conditions, the number of survived bacteria in the enriched cheeses was higher than 10^7cfu/g. The quality of the probiotic cheeses was comparable with the traditional product in respect of the dry mass, pH values, titratable acidity, NaCl content, fat content as well as nitrogen fractions. There was no significant difference in almost all quality parameters at the $p < 0.05$ level between the two probiotic cheeses.
Key words: *white brined cheese, probiotic bacteria,* Lactobacillus casei*, traditional food*

INTRODUCTION

White brined cheese, both from sheep and cow milk, is well known traditional dairy product in Balkan countries. Following the current demands for food safety, these types of cheeses are no longer produced from raw milk. Milk is pasteurized just before being used for cheese production. While consumers nowadays ask for traditional food products, they also strive for more nutritive, safer and healthier foods. Their interest in such foods has urged development of probiotic food products. Food and Agricultural Organization of the United Nations and the World Health Organization define probiotics as “live microorganisms (bacteria and yeasts), which when digested or locally applied in sufficient numbers confer one or more specified demonstrated health benefit for the host”. The use of probiotic bacterial cultures stimulates the growth of preferred microorganisms, crowds out potentially harmful bacteria and reinforces the body’s natural defense mechanisms (Anal and Singh, 2007).
The most important probiotic bacteria typically associated with the human gastrointestinal tract, belong to the lactic acid bacteria from the genera *Lactobacillus* and *Bifidobacterium*. These bacteria are Gram positive, rod-shaped, non-spore forming, catalase-negative organisms. Although the number of probiotic bacteria that delivery foods must be able to support to provide a health benefit has not been firmly established, research suggests it should be between 10^7 and 10^9 cfu/g (Sharp et. al., 2008).
Currently the probiotic cultures are successfully incorporated into large number of fermented milk products, especially in yogurts, followed by cheddar cheese, but scarcely in white brined cheese (Yilmaztekin et al., 2004; Burns et al., 2012). In recent years the consumption of cheese has increased rapidly. It is nutritious and ready-to-eat food. It has been reported that the cheese may offer certain advantages as a carrier of probiotic microorganisms, having a higher pH than yogurts and other fermented milk products (Wang et al., 2010). Furthermore, the matrix of the cheese and its relatively high fat content may offer protection to probiotic

bacteria during passage through the gastrointestinal tract (Gardiner et al., 1999; Bergamini et al., 2005).
The objective of this study was to enrich the Macedonian traditional product - the white brined cow cheese with probiotic bacterium *Lactobacillus casei* and to investigate its impact on the cheese composition during ripening.

MATERIAL AND METHODS

Cheese manufacture

The brined white cheese was manufactured from pasteurized cow's milk at industrial scale at the local cheese making company. Three batches, each of 1000 L milk were processed at the same time. A lyophilized mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* supps *bulgaricus* (Chr. Hansen Inc.) was used as a starter culture. It was dispersed in the milk according to instructions of the producer. After that, in two of the batches, a lyophilized probiotic culture of *Lactobacillus paracasei* subsp.*paracasei (L. casei)* was directly added and mixed in a dose high enough to achieve 10^{10} cfu/g (200g/1000 L) and 10^{12} cfu/g (300g/1000L). After 20 minutes, 200 ml of chymosin (Chr. Hansen Inc.) were added to each batch. The milk was allowed to coagulate for 25 min then the curd was cut (2x1.5 cm) and allowed to heal 10-15 min then heated to 35^{o}C over 15 min and cured. The curd was separated from whey at pH 6.45 and molded. The molds were first pressed with 1kg per 1kg cheese during 1.75 h, and then again with 2 kg per 1 kg cheese. The cheese was cut in smaller pieces at 30^{o}C, pH 5.4-5.62, and stored at 16^{o}C for 16 h. The cheese was brined in 20% salt solution during 3 h at pH 4.6. The young cheese was then transferred into 5% salt solution and ripened for 20 days at 16^{o}C, and for 20 days at 4^{o}C.

Chemical analysis

Cheese samples were analyzed during ripening period for moisture by drying at 105^{o}C using moisture analyzer Sartorius MA-40, salt was determined by Morh method using $AgNO_3$, titratable acidity (TA) by AOAC method 920.124, fat by van Gulick method (ISO 3433), and total nitrogen (TN) by HACH-8075 Kjeldahl method. The pH of cheese was measured with a pH-meter Mettler Toledo 120. The nitrogen fractions: water-soluble nitrogen (WSN), 12% trichloroacetic acid- soluble nitrogen (TCA) and 5% phosphotungstic acid-soluble nitrogen (PTA) were determined as described by Hayaloglu (2007).

Microbiological analysis

The population of the probiotic bacterium *Lactobacillus casei* was determined by plating sample dilutions on MRS agar and counting plate colonies after 48 h of incubation at 37^{o}C as described by Bergamini *et. al.* (2005).

Statistical analysis

Descriptive statistics and one-way analysis of variance (ANOVA) was performed on the experimental data to evaluate significant differences among the samples at 95 % confidence interval according to Tukey's test using Minitab 15 statistical software. The variables that showed high significance were used in the Principal Component Analysis (PCA) to identify a reduced number of principal components that sufficiently explain most of the information in the starting data.

RESULTS AND DISCUSSION

The gross composition of cheese samples is presented in Table 1. The table shows the mean values of moisture, pH, titratable acidity, salt content, fats-in-dray matter, nitrogen fractions soluble in water, trichloroacetic and phosphotungstic acid, and total nitrogen. Throughout the ripening period, the moisture content of the probiotic cheeses changed within narrow margins. The pH values of the samples decreased gradually during the ripening

period. At the end of the ripening period, pH of the control was 4.6, while both probiotic 1 and probiotic 2 samples had 4.4 which was a statistically significant difference. The addition of the probiotic culture affected the titratable acidity of the cheese. The acidity gradually increased during the ripening period reaching the statistically higher values of 1.6 and 1.7% in the probiotic 1 and probiotic 2 samples compared to 1.33% in the control sample. The salt penetration was fast during the first 20 days and then leveled off in both of the probiotic cheeses. After 40 days the control sample had the highest salt content.

Table 1. Chemical composition of control and probiotic white brined cheeses during ripening

Sample	control			probiotic 1*			probiotic 2**		
time (day)	3	20	40	3	20	40	3	20	40
moisture (%)	53.29±0.094^{aA}	57.00±0.343^{abA}	57.05±0.089^{aA}	51.08±0.680^{aB}	50.13±0.656^{bB}	50.16±0.163^{dB}	51.89±0.245^{aA}	49.81±0.204^{bA}	48.05±0.418^{dB}
pH	5.15±0.016^{aA}	4.48±0.392^{bA}	4.65±0.001^{cA}	5.01±0.009^{aB}	4.48±0.018^{bB}	4.39±0.003^{dB}	4.99±0.005^{aA}	4.58±0.009^{bB}	4.41±0.009^{dB}
TA (%)	0.97±0.008^{aA}	1.17±0.008^{bA}	1.33±0.005^{cA}	1.20±0.001^{aB}	1.40±0.016^{bB}	1.65±0.001^{bB}	1.26±0.009^{aB}	1.39±0.005^{bB}	1.68±0.020^{bB}
NaCl (%)	2.55±0.012^{aA}	4.67±0.010^{bA}	5.14±0.002^{cA}	2.52±0.012^{aA}	3.90±0.004^{bB}	4.04±0.004^{bB}	2.52±0.012^{aA}	3.94±0.045^{bC}	4.02±0.017^{cA}
fats (%dm)	42..86±0.444^{aA}	49.09±0.005^{bA}	51.89±0,484^{cA}	45.57±0.429^{aB}	53.02±0.412^{bB}	52..35±0.409^{bB}	48.86±0.099^{aB}	50.17±0.423^{bB}	51.50±0.392^{cC}
WSN/TN (%)	6.10±0..089^{aA}	12.83±0.197^{bA}	16.33±0.094^{cA}	6.17±0.087^{aA}	12.85±0.081^{bA}	13.89±0.085^{cB}	5.63±0.047^{aB}	11.28±0.088^{bB}	13.94±0.313^{cB}
TCA/TN (%)	2.20±0.125^{aA}	5.34±0.240^{bA}	7.64±0.045^{cA}	2.47±0.002^{aB}	6.44±0.102^{bB}	9.15±0.073^{cB}	2.51±0.070^{aB}	5.61±0..009^{bA}	6.81±0.090^{cC}
PTA/TN (%)	0.64±0.012^{aA}	1.30±0.037^{bA}	1.32±0.014^{bA}	0.75±0.016^{aB}	1.40±0.033^{bA}	1.46±0.001^{bB}	0.65±0.033^{aA}	1.36±0.023^{bA}	1.40±0.033^{bB}
TN (%)	2.70±0.016^{aA}	2.35±0.028^{bA}	2.35±0.028^{bA}	2.64±0.066^{aA}	2.76±0.001^{bB}	2.79±0.025^{bB}	2.79±0..005^{aA}	2.79±0.025^{bB}	2.91±0.034^{cC}

** Probiotic 1 refers to cheese inoculated with L. casei to achieve 10^{10} cfu/g*
***Probiotic 2 refers to cheese inoculated with L. casei to achieve 10^{12} cfu/g*
Mean values and standard deviation, (n = 3). Different small letters within a row indicate significant differences due to ripening time within the same sample ($p < 0.05$). Different capital letters within a row indicate significant differences among samples ($p < 0.05$)

The fat fraction of cheese is important for the development of typical flavor and texture. The change in the fat content exhibited in all samples may result from the salt absorption and/or water diffusion of some soluble components between brine and cheese mass (Hayaloglu, 2007). The hydrolysis of triglycerides, which constitute more than 98% of cheese fat, is the principle biochemical transformation of fat during ripening, which leads to production of free fatty acids, di- and monoglycerides and possibly glycerol. The pH of cheese has major influence on the flavor impact of free fatty acids produced by hydrolysis of triglycerides. As Sing et al. (2003) reported, at pH of about 5, a considerable portion of the fatty acids are present as salts, which are nonvolatile, thus reducing the flavor impact.

Proteolysis in cheese involves complex reactions, and to better understand the development of proteolysis in cheese, it is necessary to investigate the nitrogen fractions formed during ripening (Ong and Shah, 2008). The ratio of WSN and TCA to TN in all cheese samples increased significantly throughout 40 days of ripening. At the end of the ripening, the control sample had higher WSN fraction (16.4%) than both probiotic samples (13.9%). At the end of ripening period the PTA levels in probiotic 1 and probiotic 2 samples did not differ significantly but they showed significant difference from the control sample. The results of the increased levels of PTA fraction suggest that *Lactobacillus casei* has an active peptidolytic system. PTA fraction is composed of very small peptides, amino acids, and smaller nitrogen compounds other than dibasic amino acids and ammonia which makes the fraction fair index of free amino acid content (Bergamini et al., 2006). Total nitrogen content increased in the probiotic samples but not in the control sample.

Most publications concerning incorporation of probiotic bacteria into cheese have focused on their survival during manufacture and storage, but few studies have considered the effect of this incorporation on cheese composition and quality. The results of the principle component analysis of the chemical characteristics of the control, probiotic 1 and probiotic 2 cheese samples are presented in Figures 1-3. It can be seen that the components do not affect the overall quality of the cheese samples in same way. In general, the most pronounced effect on the quality of all cheese types had nitrogen fractions and fat content.

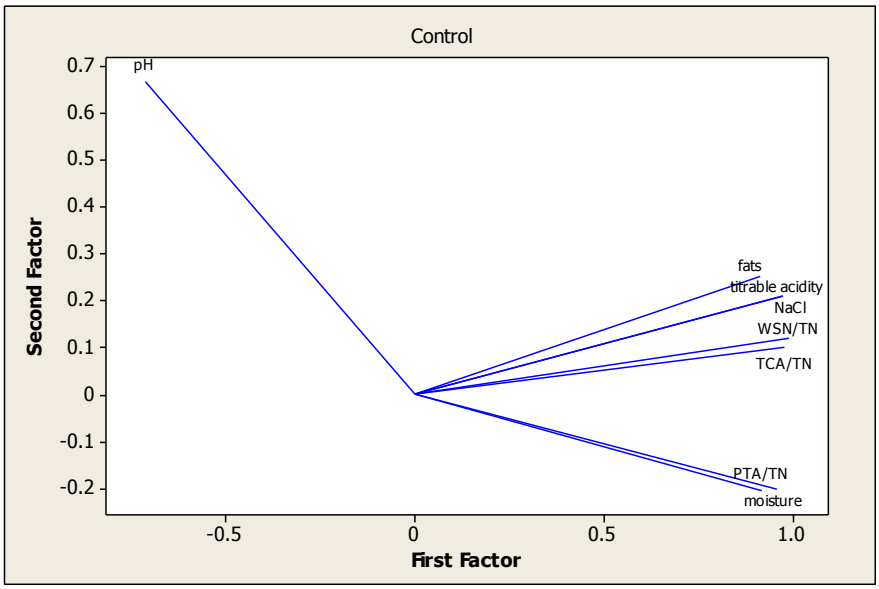

Figure 1. Principle component analysis of chemical composition of control cheese sample

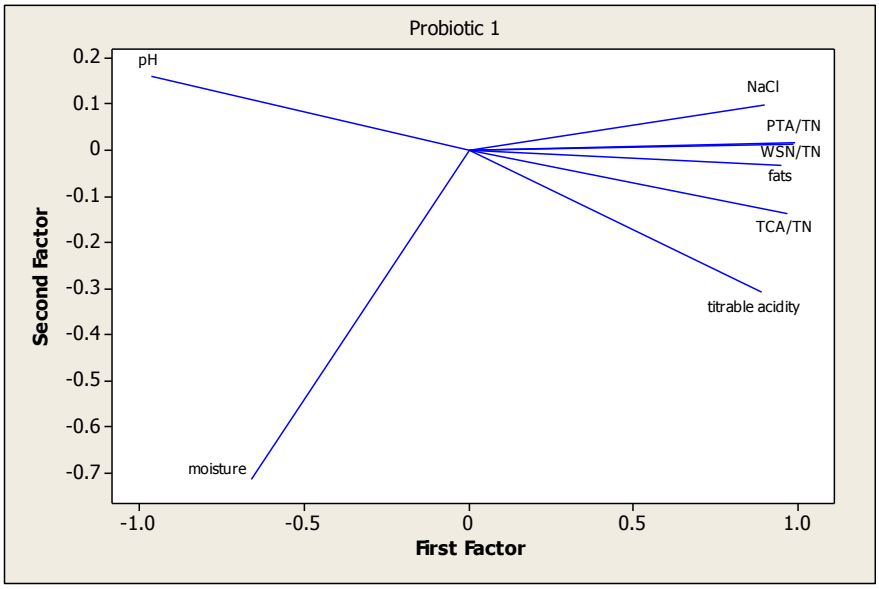

Figure 2. Principle component analysis of chemical composition of probiotic 1 cheese sample

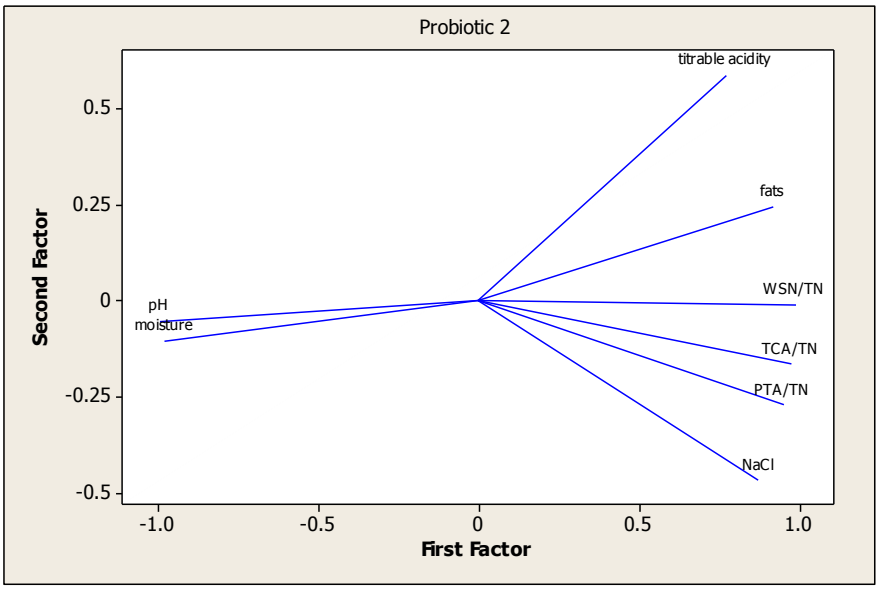

Figure 3. Principle component analysis of chemical composition of probiotic 2 cheese sample

The count of the probiotic bacteria *L. casei* during ripening of white brined cheese is shown in Fig. 4. As can be seen, in the first phase of the cheese manufacture, corresponding to the separation of the whey from the curd, there was about two log reduction in the count of probiotic bacteria in both samples. In the next period, the culture adapted to the conditions and continuously grew reaching a plateau after 30 days of ripening. The counts were about $3 \cdot 10^{10}$ cfu /g (probiotic 1) and about $5.7 \cdot 10^{10}$ cfu /g (probiotic 2). The viability of *L. casei* remained almost constant next 30 days.

Investigating the survival of *L. acidophilus* LA-5 and *B. bifidum* BB-02 in white brined cheese, Yilmaztekin et al. (2004) proved total cell viability to be from 6.8510^8 to $11.90 \cdot 10^8$ cfu /g after 30 days, and from $8 \cdot 10^7$ to $39{,}7 \cdot 10^7$ cfu /g after 60 days. Sharp et al. (2008) incorporated the lactic acid bacteria *L. casei* 334-e, an erythromycin derivative of ATCC 334, in cheddar cheese at approximately 10^7 cfu/g. The number of bacteria remained relatively constant throughout the storage period of 3 months.

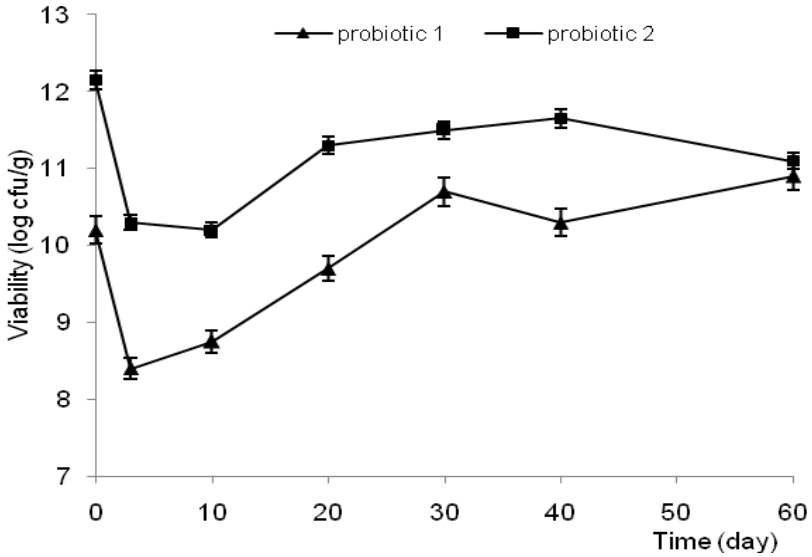

Figure 4. Viability of *L. casei* during ripening of probiotic brined white cheese

CONCLUSIONS

The traditional white brined cow cheese showed to be a very good delivery medium for probiotic culture *Lactobacillus casei*. It provided suitable conditions for the survival of the bacterium in concentrations higher than the critical concentration of 10^7cfu/g. The principal component analysis showed that nitrogen fractions and fat content are the key components influencing the overall quality of the cheeses.

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APPLICATION OF TEA FUNGUS CULTURE IN PRODUCTION OF FERMENTED MILK BEVERAGES

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ABSTRACT: Kombucha and fermented milks have again become very popular due to their positive effect on human health. The aim of this work is to investigate possible usage of tea fungus culture as a starter culture for manufacture of fermented milks. Local tea fungus culture was grown on traditional substrate at 28 ºC and 42 ºC. One group of milk samples was incubated without kombucha and another group was inoculated with 10% of local kombucha beverage. Milk-based samples were incubated at 30 ºC and 42 ºC and fermentation was stopped when the pH value of samples reached 4.5. Fermentation process was monitored by determination of basic chemical and microbiological parameters. The samples of traditional kombucha beverage incubated at 42 ºC after 4-day fermentation had the initial pH and titrabile acidity (4.61 and 0.37 g acetic acid/L) and during the first fermentation day number of acetic acid bacteria dramatically decreased from 6.6 x 10^5 (CFU/mL) to <1(CFU/mL). Pasteurized milk-based products, with and without kombucha inoculum, incubated at 42ºC reached the target pH value in almost the same fermentation time. All milk – based samples fermented at 30ºC did not reach the target pH during 24h fermentation. These results suggested that the temperature of 42 ºC is not suitable for kombucha fermentation on used traditional substrate. Lactic acid bacteria from pasteurized milk and/or environmental contaminant bacteria probably had the main role in fermentation of milk-based samples
Key words: *tea fungus, kombucha beverage, fermented milk beverages*

INTRODUCTION

Kombucha is a traditional beverage obtained by fermenting sweetened black or green tea using tea fungus culture. This beverage origins from China and the first records about it dates about 220 B.C. During the past decades, kombucha beverage became very popular in Europe and North America due to the growing awareness of general population about benefits of consuming food with functional properties.

Starter culture for kombucha fermentation is kombucha beverage, kombucha beverage with tea fungus or tea fungus. Although it is usually called fungi, microbiological it represents unique symbiosis of indigenous yeasts and acetic acid bacteria. The most common yeasts in this association belong to *Saccharomyces*, *Zygosaccharomyces*, *Saccharomycodes*, *Brettanomyces* and *Torulaspora* species. The most frequent acetic acid bacteria strains are representatives of *Acetobacter* and *Gluconobacter* species, which are part of *Acetobacteriaceae* familia.

Kombucha beverage is traditionally produced on sweetened black or green tea with sucrose. Apart from this substrate, other authors investigated possibilities for production of kombucha beverage on some alternative substrates. Until now, it is proved that fructose, glucose, lactose (Reiss, 1994), red and white wine with/without added sugar (Janković, 1995), honey (Frank, 1995), malt extract (Cvetković, 2003) and molasses (Malbaša et al., 2008) can be used as only carbon source (instead of sucrose). As far as substitutions for black or green tea are concerned, it is applicable to use sweetened tea beverage made of *Rosa canina*, mint (Janković, 1995), root and herb of *Echinacea purupurea*, as well as tea prepared of *Satureja montana* L. (Cvetković, 2003). Morales and Sánchez (2003) were the first authors who successfully applied tea fungus as a starter culture for fermentation of whey. In addition, Malbaša et al. (2009) produced fermented milk beverages using several kombucha starters.

This paper presents results of an investigation of possible usage of tea fungus culture as a starter culture for production of fermented milk beverages. Apart from that, this paper describes chemical and microbiological characteristics of kombucha fermentation process on traditional substrate at 28 °C and 42 °C as well as fermentation of milk – based samples (pasteurized and sterilized milk) at 30 °C and 42 °C.

MATERIAL AND METHODS

Kombucha fermentation: Traditional substrate for kombucha fermentation was prepared from boiled tap water with 70 g/l of sucrose and 3 g/l of black tea (Fructus, Bačka Palanka, Serbia) which was added into water after it boiled. Extraction of tea lasted 15 min and after that substrate was filtrated and obtained beverage was cooled to room temperature. Cooled tea was inoculated with 10 % v/v of previously produced kombucha beverage. Finally, every bioreactor (total volume of 720 ml, Ø 80 mm) with 330 ml of culture medium was covered with cheesecloth and put on statical incubation under aerobic condition. Traditional substrate for kombucha fermentation was incubated for 4 days at 28 °C and 42 °C. Samples for determination of chemical and microbiological parameters were taken each fermentation day and each analyze was done in two replications.

Milk fermentation

For production of fermented milk beverages two types of commercial milks were used– pasteurized and sterilized homogenized cow's milk with 2.8 % of milk fat. Both types of milk were put in bioreactors (total volume of 720 ml, Ø 80 mm) and warmed to target (incubation) temperature. After warming, samples were prepared and incubated as shown in table 1. After preparation of samples each bioreactor contained 330 ml of culture medium. All fermentations of milk-based samples were stopped when the pH value of fermentation liquid reached 4.5 and after that samples were cooled to 4 °C. One control sample with commercial yogurt starter culture (YC – 381, Christen – Hansen, Denmark) was prepared for each type of milk in order to proving the absence of substances with inhibitor effect.. Every fermentation and analyses was conducted in at least two replications.

Table 1. Plan of experiment for milk fermentation processes

Pasteurized milk with 10 % v/v of inoculum	incubated at 30 °C
	incubated at 42 °C
Pasteurized milk without inoculum	incubated at 42 °C
Sterilized milk with 10 % v/v of inoculum	incubated at 30 °C
	incubated at 42 °C

Chemical analyses

pH values of kombucha beverage and fermented milk products were measured by an electric pH-meter (HI 9321, HANNA Instruments) which was previously calibrated at values 4 and 7. Measuring of pH in samples of kombucha beverage incubated on traditional substrate was conducted after CO_2 was eliminated from sample on ultrasound bath (B-220, Branson Company, Shelton, USA) during 30 seconds. Titrabile acidity in all samples was measured with conductometric titration (OIV method, 1990). Quality carbohydrate profile was determined with thin layer chromatography with silica gel.

Microbiological analyses

All microbiological analyses were done by pour plate method. Total count of bacteria was determined by using MPA grown medium (Himedia, Mumbai, India) and incubation at 28 °C during 48h. For determination of total count of acetic acid bacteria YMP agar was used (Sievers et al., 1995) that was incubated at 28 °C at least 120h. Lactobacilli were isolated with MRS agar (Himedia, Mumbai, India) with incubation under anaerobic conditions provided by anaerobic jar with Anerocult® (Merck, Darmstadt, Germany). Lactococci were

determined with M-17 grown medium (Merck, Darmstadt, Germany). Plates for determination of lactic acid bacteria (lactobacilli and lactococci) were incubated at 37 ºC for 72h. 300 mg/l of actidion (cicloheximid, Sigma-Aldrich, St. Louis, USA) was added in all media for bacteria determination. For yeast number determination the Sabouraud Maltose Agar nutritive medium (Himedia, Mumbai, India) was used that was incubated at 28 ºC during 3 days. 50 mg/l of chloramphenicol (Sigma-Aldrich, St. Louis, USA) was added in Sabouraud Maltose Agar before inoculation.

RESULTS AND DISCUSSION

Fermentation of traditional substrate at 28 ºC and 42 ºC

In order to analyse relations between tea fungus culture and different incubation temperatures, black tea was inoculated on usual way and incubated at 28 ºC and 42 ºC. Usual temperature for kombucha fermentation is in the range 22 - 30 ºC. As Lončar et al (2006) pointed out, Petrović et al. (1995 – 1996) found that optimum temperature for kombucha fermentation is 28 °C. On the other hand, 42 ºC is temperature of yogurt fermentation, which was chosen as possible fermentation model for production of fermented milk beverages with usage of tea fungus culture (Malbaša et al., 2009). Chemical characteristics of substrate and inoculum are presented in Table 2.

Table 2. Chemical characteristics of substrate and inoculum

	sweetened black tea (substrate)		inoculum
	before inoculation	after inoculation	
pH	7.19	4.64	3.25
titrabile acidity (g/l)		0.38	5.64

Development of tea fungus fermentation at 28 ºC and 42 ºC was monitored by measuring pH and titrabile acidity (Fig. 1). Fermentation curves of sample incubated at 28 ºC showed that after 4 – day fermentation pH value decreased from 4.64 to 3.16 (1.48 pH units), while titrabile acidity (hereinafter referred as TA) increased from 0.38 to 6.46 g/l. These results are expected due to the fact that acetic acid bacteria during fermentation process produce acetic acid, gluconic acids and number of other organic acids (Reis, 1994). Comparing with results of other author, presented results for fermentation process are slightly different. For instance, after 3 – day kobucha fermentation at 24 ± 3 °C Jayabalan et al. (2007) detected 0.33 ± 0.07 g/l acetic acid and pH about 4. Apart from that, Lončar et al. (2006) in samples taken after 4-day fermentation at 30 °C detected pH 3.9 and total acid in concentration of 0.7 g/l, while in samples incubated at 22 °C after same fermentation time they measured pH of 4.55 and concentration of total acids of 0.4 g/l. However, these differences between results are quite usual because of the fact that characteristics of fermented tea depend upon the individuality of used tea fungus association (Blanc, 1996; Sievers et al, 1995; Reis, 1994).

On the other hand, fermentation process at 42 °C is quite different because during 4 – day fermentation, samples retained the initial pH and TA values. That can be explained with absence of biological activity of tea fungus culture, particularly activity of acetic acid bacteria which are dominantly responsible for production of acids (Reis 1994).

Quality sugar profile of fermentation liquid during 4 – days incubation period was monitored by TLC method (Fig 2). In sample incubated at 42 °C after 24h glucose was detected in very small amount, while in the samples incubated at 28 °C this amount is much higher. By the end of 4 - days process, amount of sucrose in both substrates was reduced, but in samples incubated at 28 °C that process happened more intensively. These results are consistent with results of other authors (Lončar et al, 2006; Sievers et al 1995) who proved that during kombucha fermentation concentration of sucrose steadily reduce and concentration of glucose increase. This happens because the first step in kombucha metabolic pathway is extracellular hydrolysis of sucrose to fermentable glucose and fructose.

Microbiological profile of kombucha beverage during 4 – days fermentation at 28 °C and 42°C is shown in Fig 1. In samples incubated at 28 °C total count of acetic acid bacteria during 4 – day fermentation increased for about 1 log unit, while the count of yeasts remained at almost the same number (at about 6 log units). Total count of yeasts in samples incubated at 42 °C after the fourth fermentation day was 1.2 x 10^6 (CFU/ml), which was slightly different compared with sample incubated at 28 °C (5.1 x 10^6 (CFU/ml)). Comparing with the results of other authors, a good correlation can be noticed. For example, Sreeramulu et al. (2000) at the fourth fermentation day detected yeasts and acetic acid bacteria in count of about 7 – log units. Chen and Liu (2000) at the sixth fermentation day detected total count of acetic acid bacteria in average about 10^3 (CFU/ml or g) and total count of yeast of about 10^6 (CFU/ml or g).

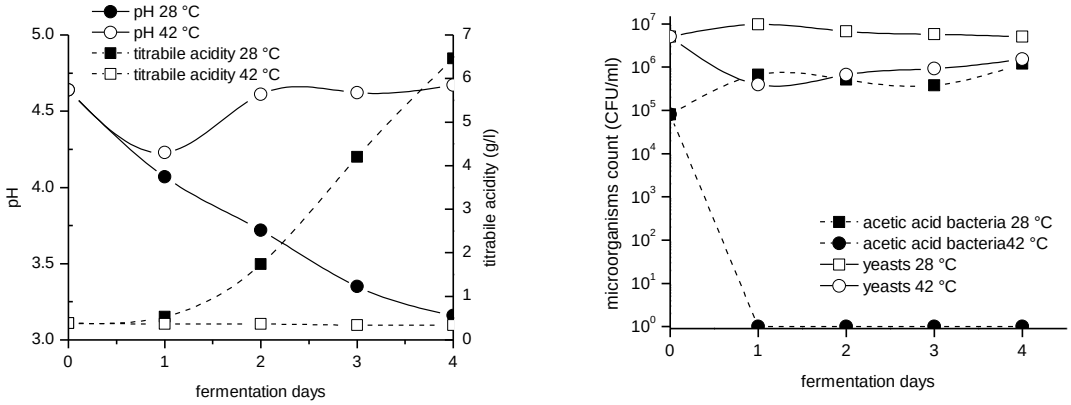
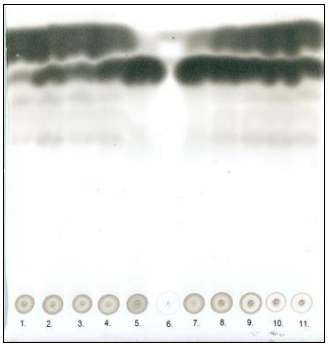


Figure 1. Basic chemical and microbiological parameters of kombucha during fermentation process

On contrary, the total count of acetic acid bacteria in sample incubated at 42 °C during the first fermentation day reduced from initial count to <1 CFU/ml and stayed at that level until the end of fermentation. In addition to that, sample incubated at 42 °C did not have cellulose pelicule on the surface, which is a typical metabolite of acetic acid bacteria and which was produced in samples incubated at 28 °C. This phenomenon can be explained with the fact that optimum temperature for most of acetic acid bacteria is 25 – 30 °C, while the maximum temperature is below 42 °C.



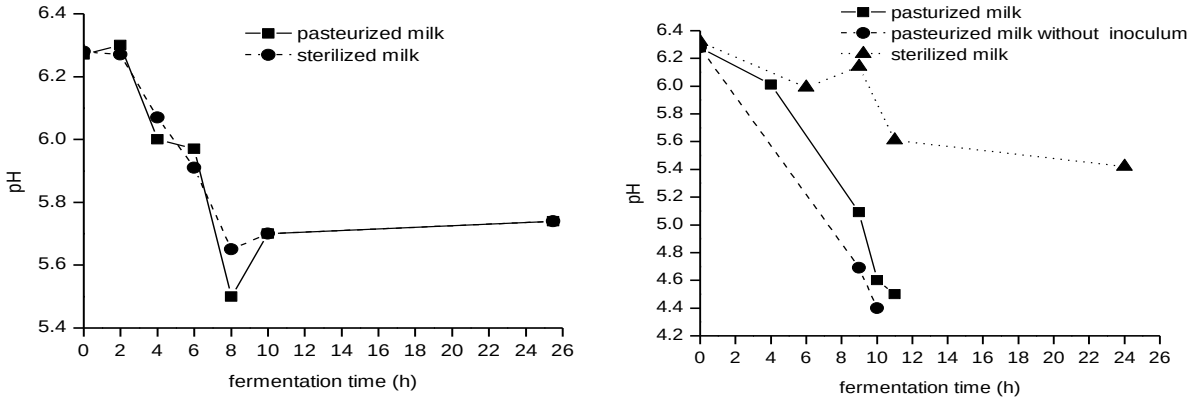
1) 4. day at 28 °C
2) 3. day at 28 °C
3) 2. day at 28 °C
4) 1. day at 28 °C
5) substrate before inoculation
6) standard mixture (sucrose + glucose)
7) 1. day at 42 °C
8) 2. day at 42 °C
9) 3. day at 42 °C
10) 4. day at 42 °C
11) inoculum

Figure 2. TLC chromatogram of samples incubated at 28 °C and 42 °C

Fermentation of milk – based samples at 30 °C and 42 °C

The development of all fermentation processes of milk – based samples are presented in Fig. 3. All milk – based samples did not contain inhibitor substances because in all control samples fermented milks were produced during the time declared by producer of commercial starter culture. As can be noticed in the Fig 3., all milk – based samples (pasteurized and sterilized) incubated at temperature of 30 °C did not reach the target pH value and during 26h fermentation process pH was only reduced for 0.54 pH units (from 6.28 to 5.74). Results for fermentation process of sterilized milk incubated at 42 °C are similar because after 22h of fermentation pH was still about 5.7 (reduced only for only 0.57 pH units). Although lactose

(as the main carbohydrate source) was presented in these samples, it is obvious that microorganisms did not use it for biotransformation and intensive production of acids.
Results for pasteurized milk incubated at 42 °C are different because fermented milk beverage with pH 4.5 was produced during 10.5h. Our previous work showed that usually fermentation times for milk – based samples with kombucha inoculum are in range 10.5-16h (Ivković, 2011). Comparing with results of other authors, it can be concluded that presented fermentation processes are slower. Malbaša et al. (2009) produced fermented milk beverages with usage of concentrate of kombucha during 6–6.5 h.
Interesting results were gained for samples incubated without kombucha inoculum because the time and trend of milk fermentation was almost the same as for samples inoculated with kombucha beverage.



(a) (b)

Figure 2. Fermentation curves for milk – based samples incubated at 30 °C (a) and 42°C (b)

In Table 3 are presented results of microbiological analyses of produced fermented milk beverages. Dominant microorganisms in microbe association of produced beverages were lactic acid bacteria (lactococci and lactobacilli). They were probably detected in final product in that number because they represented initial contamination of milk or they were environmental contaminants. Initially, milk contained 1.3 x 10^3 (CFU/ml) lactococci, 3.2 x 10^2 (CFU/ml) lactobacilli and 2.4 x 10^3 (CFU/ml) of total bacteria. As can be seen, the biggest increase is detected by lactobacilli (almost 6.5 log units), while lactococci had increase of almost 6 log units. On YMP media, growth of 1.2 x 10^3 (CFU/ml) was detected. However, microbiological analyses of colonies from YMP media showed that present microorganisms were Gram positive and spherical (cocci) bacteria, not Gram negative and rod – shaped, as was expected for acetic acid bacteria. That means that YMP media is not adequate for determination of acetic acid bacteria in samples of fermented milks.

Table 3. Microbiological profile of fermented milk beverages

fermented beverage	**total count of bacteria (CFU/ml)**	**yeasts (CFU/ml)**	**lactobacilli (CFU/ml)**	**lactococci (CFU/ml)**
pasteurized milk with kombucha inoculum	4.8 x 10^7	6.8 x 10^5	9.3 x 10^8	1.4 x 10^9
pasteurized milk without kombucha inoculum	2.7 x 10^7	< 10	1.1 x 10^9	1.9 x 10^9

These results are in certain correlation with results presented by Iličić (2010). In her analyses she concluded that in fermented beverages produced on milk with 0.9 % of milk fat with usage of kombucha culture 80 % of microorganisms are lactic acid bacteria, 10 % are yeast and 10 % are acetic acid bacteria.

CONCLUSIONS

According to these results it can be concluded that acetic acid bacteria lose viability during the first fermentation day at 42 °C and that was the reason why no changes in acidity (tritrabile acidity and pH) of fermented products were detected. On the other hand, yeast from tea fungus, after short adaptation period to 42 °C, continue with their physiological activities which can be detected by reduction of sucrose concentration in traditional substrate and increased total count of yeasts.
According to results for milk – based samples, it can be concluded that kombucha culture is not suitable as a starter culture for fermentation of sterilized milk. Although 30 °C is usual temperature for kombucha fermentation, application of kombucha in milk – based samples at that temperature is not showed as appropriate in this work.
On contrary, in samples of pasteurized milk with addition of kombucha inoculum incubated at 42°C fermented milk beverages were produced. Due to the fact that samples without kombucha inoculum also reached target pH in almost the same time as samples with kombucha inoculum and according to microbiological analyses of final products, it can be assume that fermentation is caused by lactic acid bacteria from pasteurized milk and/or environmental contaminant bacteria.

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FIRST INSIGHT INTO THE ANTIOXIDANT ACTIVITY OF SUGAR BEET INTERMEDIATE PRODUCTS

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ABSTRACT: While the nature of antioxidants in sugar cane processing is well documented and understood, little information exist about the antioxidant activity of sugar beet manufacturing products. In this work, besides basic quality parameters, antioxidant activity of A (white) sugar, B (raw) sugar and affinated C sugar, obtained from the process of sugar production was tested. As the antioxidant activity of cane brown sugars was connected with the presence of polyphenol compounds and Maillard reaction products, the content of these compounds was measured, as well.
Apart from the highest sucrose content (99.50 °Z), A sugar was characterized with the lowest invert sugar (0.008%/DM) and coloured matter content (54 IJ/DM). However, A sugar showed no antioxidant activity and also it did not contain a detectable amount of plant phenolics. Contrary to this, sugars B and C contained less sucrose (99.22 and 97.6 °Z, respectively) and more coloured matter (329 and 20366 IJ/DM, respectively). Antioxidant activity of C sugar was higher than that of B sugar, which corresponded with higher total phenolic and coloured matter content of C sugar.
Our results indicate that both, plant phenolics and Maillard reaction products can be associated with the antioxidant activity. Despite the fact that colour of the final product presents a key quality measurement in sugar industries, with regard to the antioxidant activity, consumption of brown sugars can be recommended.
Key words: *sugarbeet, A (white) sugar, B (raw) sugar, affinated C sugar, antioxidant activity*

INTRODUCTION

Colour of the final product is a key quality measurement in sugar industries. While the nature of colouring substances in sugarcane processing is well studied and documented (Bento and Sá, 1998), colour formation in sugar beet manufacture is not much examined and understood. Because of that, it is not possible to predict the content of coloured substances in the final product on the basis of raw material chemical composition. Coloured substances are formed through beet processing as a result of pH changes, thermal and autocatalytic effects. These impurities are of high molecular masses, polymeric and with tendency to occlude within the sugar crystal (Coca et al., 2008). Maillard reaction and alkaline degradation of invert sugars have been considered to be the main mechanisms of colour formation during purification stage (Coca et al., 2008). Melanoidins, as the type of Maillard reaction products, are formed by the reaction of monosaccharides and carbonyl compounds with amino acids, and are recognized as being acidic and polymeric compounds, with a highly complicated structure (Cämmerer and Kroh, 1995; Cämmerer et al., 2002). According to Heitz (1995), up to 80% of colour in sugar beet juice is a result of alkaline degradation of hexoses (HADP). The production of colored HADP takes place at the common pH of a beet sugar factory (8–11) while the formation of degradation products occurs mainly in the purification step where temperature increases up to 85 °C and pH rises up to strong basic values (11–12). The nature and structure of colored HADP have not been elucidated but they are probably due to the extensive aldolisation of intermediate dicarbonyl compounds in alkaline solutions (de Bruijn, 1986). Coloured substances formed through sugar beet processing negatively influence the quality of white sugar. However, Maillard reaction compounds have been reported to possess antioxidant activity (Yanagimoto et al., 2002; Yanagimoto et al., 2004). Payet et al. (2005) reported the antioxidant activity of cane brown

sugars, which was connected with the presence of polyphenol compounds and Maillard reaction products. As the concentration of nitrogen compounds in sugar beet juices is higher than in cane sugar juices, Coca et al. (2004) assumed that the formation of melanoidins in sugar beet processing was more intensive.
The objective of this work was to investigate antioxidant activity of A (white) sugar, B (raw) sugar and affinated C sugar and the molecules that might be responsible for the activity, phenolic compounds, and Maillard reaction products.

MATERIAL AND METHODS

Materials

A (white) sugar, B (raw) sugar and affinated C sugar were obtained from the Sugar factory „Šajkaška“, Žabalj, Serbia. The basic characteristics of the samples were determined according to the methods specified by Reinefeld and Schneider (1978).

HPLC determination of fructose, glucose, raffinose and sucrose

A high-performance liquid chromatographic (HPLC) method was applied to determine fructose, glucose, raffinose and sucrose content of samples previously dissolved in distilled water and diluted to 40°Bx dry matter. The HPLC (HP 1090, Hewlett-Packard, USA) system consisted of a Zorbax Carbohydrate 4.6 × 250 mm, 5 µm column, a solvent system of acetonitrile/water, 70/25 (v/v), a flow rate of 1 ml/min, and a refractive index detector operating in the range od 4×10^{-5} RIU and at the temperature of 35 °C. Prior to injection, all samples were properly diluted in deionised water and filtered through a 0.45 µm Rotilabo-Spritzenfilter CME (Carl Roth).

Scavenging activity on DPPH$^{\cdot}$

Samples were dissolved in deionized water, in an appropriate manner to obtain a series of dilutions. Scavenging activity on DPPH$^{\cdot}$ (1,1-diphenyl-2-picryl-hydrazyl radical) was determined spectrophotometrically following the procedure of Espin and others (2000). The IC_{50} (mg/mL) was defined as the mass concentration of an antioxidant extract which was required to quench 50% of the initial DPPH$^{\cdot}$ under the given experimental conditions. It was obtained by interpolation from linear regression analysis.

Determination of total phenolic content

Total phenolic content of samples, previously dissolved in distilled water and diluted to 40°Bx dry matter, was determined spectrophotometrically at 750 nm by using Folin-Ciocalteu's reagent (Singleton et al., 1999). Gallic acid was used as a standard and results were expressed as gallic acid equivalents (GAE) (µg GAE per g of sample).

Statistical analysis

All analyses were performed in triplicate, and the mean values with the standard deviations are reported. Statistical data analysis software system STATISTICA (StatSoft, Inc. (2011). data analysis software system, version 10.0. www.statsoft.com) was used for analysis.

RESULTS AND DISCUSSION

Basic quality parameters of A (white), B (raw) and affinated C sugars are presented in Table 1. Obtained values are as expected for each of the investigated sugars.

Table 1. Basic quality parameters of A (white), B (raw) and affinated C sugars

	A (white) sugar	B (raw) sugar	C (affinated) sugar
Dry matter content (%)	99.97	99.89	99.82
Sucrose content (°Z)	99.50	99.22	97.6
Invert sugar content (%/DM)	0.008	0.083	0.197
pH value	6.40	6.67	6.81
Coloured matter content 420 nm (IJ/DM)	54	329	20366

The contents of sucrose, fructose, glucose and raffinose in the investigated samples diluted to 40°Bx dry matter are presented in Table 2. In comparison with other investigated samples, A sugar had the highest content of sucrose and the lowest content of fructose and glucose. The content of raffinose in A sugar solution at 40°Bx dry matter was below the limit of detection of the method, while it was higher in affinated C sugar than in raw B sugar. The content of glucose and fructose of C sugar was higher than of B sugar.

As the antioxidant activity of cane brown sugars was connected with the presence of polyphenol compounds and Maillard reaction products (Payet et al., 2005), apart from coloured matter content at 420 nm (Table 1), the total phenolic content of investigated samples was determined (Table 3). The total phenolic content of C sugar was significantly higher than of B sugar, while in the case of A sugar it was below the detection limit of the method. The same stands for the coloured matter content at 420 nm, indicator of Maillard reaction extend. The coloured matter content of C sugar was 62 times higher than of B sugar and 377 times higher than of A sugar.

Due to its convenience, DPPH$^{\cdot}$ scavenging activity test is frequently used to estimate the antioxidant activity of different food matrices.

Table 2. The content of sucrose, fructose, glucose and raffinose in A (white), B (raw) and affinated C sugars at 40°Bx dry matter

Sample	Sucrose (mg/ml)	Fructose (mg/ml)	Glucose (mg/ml)	Raffinose (mg/ml)
A 40 Bx	459.8 ± 2.91	0.25 ±0.022	0.22 ± 0.09	-
B 40 Bx	456.8 ± 3.35	1.10 ± 0.180	1.17 ± 0.28	1.45 ± 0.18
C 40 Bx	450.9 ± 1.68	1.75 ± 0.110	1.97 ± 0.17	2.10 ± 0.25

Scavenging activity against DPPH radicals positively correlated with the total phenolic and coloured matter content of the samples (Table 1. and 3.). Higher scavenging activity corresponds to lower IC_{50} value. As in the case of total phenolics, DPPH$^{\cdot}$ scavenging activity of A sugar could not be established. According to obtained results, both plant phenolics and Maillard reaction products can be associated with the antioxidant activity.

Table 3. Total phenolic content and DPPH$^{\cdot}$ scavenging activity of the investigated samples

Sample	DPPH$^{\cdot}$ scavenging activity IC_{50} ± SD (g/ml)	Total phenolic content mg G.A.E /g
A (white) sugar	-	-
B (raw) sugar	0.676 ± 0.170	0.012 ± 0.001
Affinated C sugar	0.440 ± 0.026	0.494 ± 0.090

CONCLUSIONS

Based on the preliminary results of this experiment, sugar refinement in sugar beet industries, apart from reduction of coloured matter and total phenolic content, leads to drastic decrease in antioxidant activity of the final product (white A sugar). Despite the fact that colour of the final product presents a key quality measurement in sugar industries, with regard to the antioxidant activity, consumption of brown sugars can be recommended.

ACKNOWLEDGEMENTS

This work is a part of the Project (TR-31014) supported by the Ministry of Education and Science, Republic of Serbia.

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ETHANOL PRODUCTION FROM HOMINY FEED - BYPRODUCT FROM CORN MILLING

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ABSTRACT: Hominy feed (HF) is a by-product generated by the manufacture of corn meal and is similar to ground corn in appearance. The initial composition (db) of HF is around 60% starch, 14% fibre, 12% fat, 10% protein and 4% cellulose. The market competitiveness and price of HF can be boosted by fermentation of HF starch into ethanol. Before fermentation HF has to be defatted (HF_{DF}). Saccharification and fermentation were performed by three different methods: application of ultrasounds after enzymes addition (US), addition of $CaCl_2$ (Ca) and simultaneous application of two enzymes, α-amylase and glucoamylase, on double quantity (DE). These three different methods, plus a control, were tested using first hydrolysis followed by fermentation (SHF) and in simultaneous saccharification and fermentation (SSF). Fermentation was performed by *Saccharomyces cerevisiae*, at 10^8 cfu/ ml. After 48 h of fermentation of 30% HF_{DF} (% W/V) aqueous solution, ethanol was the main product (from 62 to 97 g/ L), and glycerol (from 8 to 10 g/ L) and acetic acid (from 0.7 to 1.4 g/ L) the secondary products. Highest level of glycerol occurs in simultaneous with highest concentration ethanol, experiments coded DE/HSF and Ca/SSF. Highest level of acetic acid occurred at lowest concentration ethanol in the ultrasonicated experiments. In conclusion, ethanol yields varied from 260 to 500 l/ton HF_{DF}, on the control/SHF and Ca/SSF experiments, respectively. The highest hydrolyzed starch conversion factor into ethanol was 90%. HF showed to be a good sugar source to produce bioethanol.
Key words: *Hominy feed, Ethanol, Separate hydrolysis and fermentation, Simultaneous saccharification and fermentation*

INTRODUCTION

Humankind faces important decisions related to mitigate the impacts of climate change, and reduce greenhouse gas emissions (GHG's) (Tat Tan *et al*., 2008 and Scacchi *et al*., 2010). This situation could be dramatically improved by using renewable and more sustainable fuels to replace fossil fuels (Gnansounou, 2010). Coppola *et al*. (2009) predicts that the European market will increase the consumption of biofuels from its current 2%, up to 25% by 2030. This was the subject of a European Directive n.º 2003/30/CE of 8th May.
Directives of governments accelerated the interest in biofuels, including time bound targets for biofuels consumption (Anonymous, 2009).
In general, biofuels like bioethanol and biodiesel, are produced from agricultural products, e.g. corn, sugarcane, palm oil, rapeseed or soybean. These biofuels, produced from food sources, are known as first-generation biofuels (Tat Tan *et al*., 2008). Biofuel development and production could have a high impact on the structure and distribution of agricultural production, agricultural trade and the welfare of different households (Qiu *et al*., 2010).
Demand for biofuels feedstocks increased world food prices for food and feed cereals by some 140 %, between 2002 and 2007 (Anonymous, 2009).
However, this situation can be minimized if biofuels are produced from the low cost industrial by-products. In today's market, hominy feed (HF) is a cost-effective by-product and represents about 35% of the corn industry production volumes.

In the present study, the HF comes from a protuguese corn milling industry. The conversion of starch into ethanol was evaluated by three different methods using first hydrolysis followed by fermentation (SHF) and in simultaneous saccharification and fermentation (SSF).

MATERIAL AND METHODS

Hominy feed composition

Hominy feed (HF) was obtained from corn milling industry in Torres Vedras – Portugal. Original HF was analyzed in duplicate by Biopremier Laboratories (Lisbon, Portugal). The samples were analyzed for moisture (method PAQF 120.2), starch (method BOE207748), oil (NP 876: 2004), fibre (MI LAQ 103.02), protein (method PAFQ 360.1) and cellulose (method EN ISO 6865: 00).

Oil extraction

Before oil extraction the HF was grounded and was granulometricaly separated to obtain a fraction with 0.5 mm of diameter, to increase the yield of oil extraction. The HF was defatted by Soxhlet extraction with hexane, during 8 h at 80 ºC into HF_{DF} with residual oil.

Fermentation

Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) were conducted to produce ethanol, both combined with the application of ultrasounds after enzymes addition (Mojović et al., 2006 and Nitayavardhana et al., 2010), enrichment with 60 ppm of Ca^{2+} (as $CaCl_2$. H_2O) (Merk, Germany) (Mojović et al., 2006) and double the amount of enzymes (Table 1).

Table 4. Samples codification

	Control	Ultrasound	Ca2+	Double of amount of enzymes
SHF	SHF_Control	SHF_US	SHF_Ca	SHF_DE
SSF	SSF_Control	SSF_US	SSF_Ca	SSF_DE

SHF was carried out by adaptation of work of Mojović (2006) and Sharma (2008), HF_{DF} was mixed with water to obtain 30 % solid content in the slurry. The mixture was treated with enzymes in two steps. The first step, liquefaction was performed at 85 ºC during 1 h at pH 6 (Sharma et al., 2008 and Mojović et al., 2010) with α-amylase (0.5 % v/v) (Liquozyme Supra – Novozymes), and the second step, saccharification, was performed at 55 ºC, 4 h, pH 5 (Sharma et al., 2008 and Mojović et al., 2010) with glucoamylase (0.25 % v/v) (Optimax 4060 VHP, Genencor). The mash was subject to ethanol fermentation by Saccharomyces cerevisiae ISA 1000 (PYCC 4072) (10^8 ufc/ ml), at 30 ºC during 48 h, with mixing rate 350 rpm.

SSF was conducted by protocol of Sharma (2008), similar conditions were used for SHF, but with hydrolysis, saccharification and fermentation at same time.

For HPLC analysis, (Sharma *et al.*, 2008) the mash samples were split into two 1.5 ml samples and centrifuged for 10 min at 12000 rpm, to obtain clear supernatant liquid. Supernatant was passed through a 0.2 µm sterile syringe filter into 1 ml shell vial. Filtered liquid was injected into a chromatography column (Shodex SH 1011, Waters, USA) maintained at 50 ºC. HPLC grade water containing 5 mM sulphuric acid was used as a mobile phase. Elution rate was 0.6 ml/ min. Separated components were detected with a refractive index detector (2410 – Waters, USA). Data were processed using HPLC software (Empower 2).

RESULTS AND DISCUSSION

Hominy feed composition

The composition of hominy feed show in Table 2 is compared with reference values. Ash value was within the range reported by Sharma *et al.* (2008). Protein and starch values are slightly lower than the range reported in the literature, while values of fat and fiber are higher (Raush and Belyea, 2006 and Sharma *et al.*, 2008).

Table 5. Centesimal composition of hominy feed (HF), in dry basis, with 10.3 % ± 0.8 of moisture

	% Dry basis ± SD	Reference values
Ash	3.0 ± 0.1	0.7 - 3.2
Protein	9.8 ± 0.66	9.6 - 12.3
Fibre	13.7 ± 9.3	6.7
Starch	59.6 ± 10.8	56.9
Cellulose	4.3 ± 2.9	____
Fat	12.5 ± 0.2	2.7 - 8.3

Oil Extraction

The optimized oil extraction led to 12 % (in dry basis) of oil. By comparison with results presented before we can observe that yield of oil extraction is close to 100 %.

Ethanol Yield

In SHF it was possible to measure the amount of released glucose. In our conditions we can achieve a yield of glucose extinction between 110 and 130 g/ L (data not shown), at the end of saccharification. This value was higher than value achieved by Mojović (2010) and within the range reported by Khanal (2007), 70g/ L and 160 g/ L.

During SSF the glucose content in the fermentation mash was undetected after 24h indicating rapid sugar utilization by yeasts.

After 48 h of fermentation ethanol yields of 7.8 and 12.5 % (v/v) were obtained from the hominy feed (figure 1). The highest ethanol yields were achieved by samples DE_SSF and Ca_SSF, respectively 12.1% and 12.5% of ethanol (v/v). The lowest ethanol yield was from sample control_SHF (7.8%). The highest values showed a small difference between them, however, if we express the results in L ethanol/ ton HF_{DF} we obtain for DE_SSF and Ca_SSF samples, respectively, 444 L/ ton HF_{DF} and 500 L/ ton HF_{DF}, that correspond to an increase of 11% in yield.

The highest values of ethanol were obtained for SSF, these values were higher than values achieved by Sharma (2008), maximum 380 L/ metric ton, and similar to concentration found by Brethaven (2010), 11 – 12% v/v.

Simultaneously, the secondary products of fermentation were measured, glycerol (Figure 2) and acetic acid (Figure 3), to monitor conditions of fermentation. The range of glycerol was 0.67 – 0.82 % (v/v) and acetic acid 0.07 – 0.14 % (v/v).

Comparing our results with the values found by Sharma (2008), the value of acetic acid was in accordance (< 0.5 % w/v) but the value of glycerol was much higher than the values found by them (< 0.4 % w/v).

Highest level of glycerol occurs in simultaneous with highest concentration of ethanol, i.e. experiments coded DE/HSF and Ca/SSF. Highest level of acetic acid occurred at the lowest concentration of ethanol, in the ultrasonicated experiments.

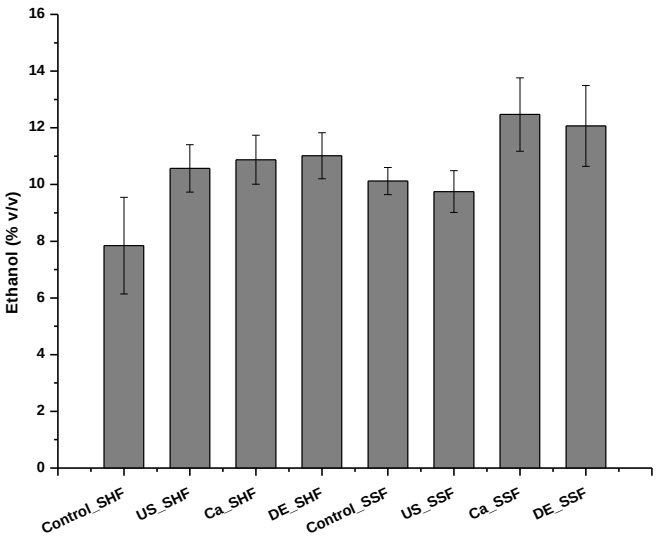

Figure 3. Final ethanol concentration for each tested conditions in separate (SHF) and in simultaneous (SSF) hydrolysis, saccharification and fermentation: control (control_SHF; control_SSF), application of ultrasounds after enzymes addition (US_SHF; US_SSF), addition of $CaCl_2$ (Ca_SHF; Ca_SSF) and application of the double amount of enzymes (DE_SHF; DE_SSF).

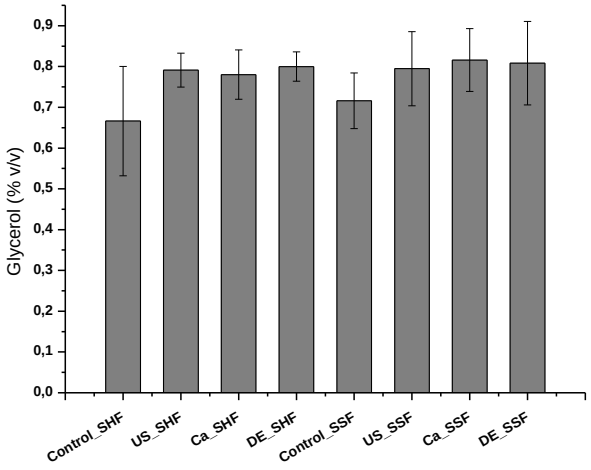

Figure 4. Final glycerol concentration for each tested conditions in separate (SHF) and in simultaneous (SSF) hydrolysis, saccharification and fermentation: control (control_SHF; control_SSF), application of ultrasounds after enzymes addition (US_SHF; US_SSF), addition of $CaCl_2$ (Ca_SHF; Ca_SSF) and application of the double amount of enzymes (DE_SHF; DE_SSF).

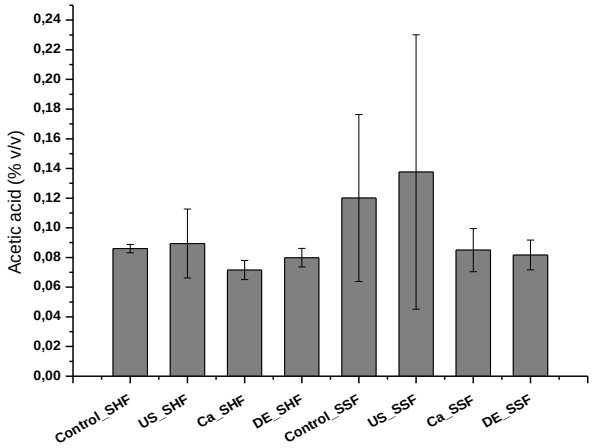

Figure 5. Final acetic acid concentration for each tested conditions in separate (SHF) and in simultaneous (SSF) hydrolysis, saccharification and fermentation: control (control_SHF; control_SSF), application of ultrasounds after enzymes addition (US_SHF; US_SSF), addition of $CaCl_2$ (Ca_SHF; Ca_SSF) and application of the double amount of enzymes (DE_SHF; DE_SSF).

CONCLUSIONS

By centesimal composition, hominy feed have a high value in oil and starch content that could be converted into ethanol. The ethanol yields that were obtained varied from 260 to 500 l/ton HF_{DF}, on the control_SHF and Ca_SSF experiments. These values show that hominy feed has a great potential to be used as a feedstock for ethanol production, and is of secondary source, not competing with food crops.
In conclusion, ethanol yields varied from, 444 L/ ton HF_{DF} to 500 L/ ton HF_{DF} . The highest hydrolyzed starch conversion factor into ethanol was 90%.

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SENSORY AND INSTRUMENTAL EVALUATION OF QUALITY ATTRIBUTES OF COOKIES ENRICHED WITH MEDICINAL PLANT MIXTURE

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ABSTRACT: Increasing health-consciousness among consumers has boosted their interest in added value food products. As a source of biologically active substances including antioxidants and antimicrobials, medicinal plants can be considered as functional food ingredients. In this research, new cookie formulations were developed by supplementing the basic cookie formula with a mixture of medicinal plants, composed to promote digestion. The objective of this study was to investigate the effects of addition on the quality properties of cookies. The medicinal plant mixture was included in cookie formulations at three levels (2%, 4%, 6%) and compared to the control, which was based on wheat flour. Referring to the results, medicinal plant addition caused statistically significant ($P < 0.05$) differences in sensory and instrumentally measured colour and texture parameters of the cookies. Based on their overall good sensory acceptability and previously confirmed antioxidant and antimicrobial properties of the mixture, new cookie formulations can broaden the utilization of this mixture and may be regarded as health-promoting functional foods.
Key words: *cookies quality, medicinal plants, sensory evaluation, colour, texture*

INTRODUCTION

Development of new products by adding functional ingredients to carrier food provides not only new market opportunities for food producers, but also potential benefits for consumers (Krutulyte et al., 2011). Short dough cookies are widely consumed food products, appreciated for their versatility, convenience, conservation, and especially for their attractive sensory attributes (Verbeke et al., 2009), and for these reasons they can be considered to be suitable carrier products for functional bakery formulations. However, there is no previous research on sensory evaluation of specific carrier-ingredients combinations such as cookies enriched with medicinal plant mixture. As a source of biologically active substances including antioxidants and antimicrobials, medicinal plants can be considered as functional food ingredients (Mišan et al., 2009). Their potential application arises from the fact that they contain wide area of secondary biomolecules and pharmacologically active constituents, such as essential oils, antimicrobial substances, which can enhance flavour at the same time, plant phenolics, which are powerful antioxidants, organic acids, resins, pigments, vitamins and minerals (Willbrandt, 1989). However, the addition of some functional ingredients could result in changes in the sensory attributes which may lead to changes in consumers' acceptance (Glanz et al., 1998). Concerns about the reductions in taste quality can interfere with the adoption of healthy diets, since consumers emphasize sensory experiences during consumption (appearance, texture, flavour), with the pleasure derived from consumption as an important motivator in eating (Westenhoefer and Pudel, 1993). In respect of the above facts, descriptive sensory analysis remains an important method in the evaluation of different foods, including cookies which are usually applied before the usual consumer tests (Stone and Sidel, 1998). When integrated within the product development process, sensory testing allows cost-effective delivery of acceptable products to consumers and thus reduces the risk of failure (Lawless and Heymann 1998).

Referring to all mentioned above, the aim of this research was to evaluate the ability of sensory evaluation, instrumental colour and texture analysis as descriptive quality attributes of cookies.

MATERIAL AND METHODS

Materials

The commercial plant mixture 'Vitalplant' was obtained from the Institute for Medicinal Plants Research 'Dr Josif Pančić' (Belgrade, Serbia). It was provided in three packages, each containing 500 g of material. The mixture was in the form of powder with granulation of up to 3 mm. Presence of herbal drugs in the composition of the 'Vitalplant' mixture was: *Frangulae cortex* 35%, *Petroselini fructus* 25%, *Menthae pip. folium* 20%, and *Carvi fructus* 20%.

Preparation of cookies

Bake trials were conducted under laboratory conditions. Dough mixing, processing and baking were performed on laboratory-scale equipment. The basic cookie dough formulation (control sample) contained 100 g of refined wheat flour, 40 g of vegetable fat, 30 g of powdered sugar, 1 g of salt, 1 g of baking powder. Plant mixture was added at three doses 2, 4, and 6% (flour basis). The amount of water added varied to obtain cohesive dough suitable for the production of mold-cut cookies. Ingredients were mixed in Diosna mixer as per "all-in" method. All ingredients were mixed together in one phase for 15 min. The formed dough was packed in polyethylene bags and left to rest 0.5 hour at ambient temperature (22-24ºC Following the rest time, the dough was sheeted to a final thickness, approximately 5 mm on a pastry break, and cut out using a rectangular cutter. Dough pieces were baked for 12 min in a deck oven at 170°C. After cooling, the cookies were placed in polyethylene bags and stored at ambient temperature until further examination (Mišan et al., 2009).

Physical characteristics and sensory evaluation

Cookies were evaluated for different parameters. Five, randomly chosen cookies were taken to measure width and thickness. Spread was calculated from the ratio of width and thickness. Sensory evaluation was performed 24 h after baking by six experienced panellists (35-50 years old). The samples were evaluated on two occasions. Sensory profiling was performed using a generic descriptive analysis technique, included the selected representative attributes of cookies (Tang, et al., 1999; Sikora et al., 2007). The attributes were evaluated using a 5-points method. Each mark was described with words, using previously prepared standard cards (Pajin, 2009; Sikora et al., 2007; Sedej et al., 2011). In order to obtain the overall cookies quality, prior to sensory evaluation, importance coefficients (IC) for each sensory attribute were standardized and fixed by the panel. The importance coefficients were balanced that their sum equals to 4.0. The obtained marks were multiplied by the following importance coefficients: IC = 0.8 for appearance (shape and surface); IC = 0.8 for structure, and break; IC = 0.8.0 for chewiness; IC = 0.6 for odour; and IC = 1.0 for taste. Applying the importance coefficients, a quantitative expression of the total product quality is obtained as the 'weighted' mean value of the scores for each evaluated attribute.

Cookie surface colour measurements

Surface colour of cookies was determined by chromameter MINOLTA, CR-400 (Minolta Co., Ltd., Osaka, Japan) with illuminate D-65, and standard observer function 2°. Colour values of top and bottom surfaces, at five measured points (centre and at the corners of cookies), were recorded for 10 randomly chosen cookies per batch and averaged. The results were expressed as lightness (L^*), redness-greenness (a^*), and yellowness-blueness (b^*), according to CIE $L^*a^*b^*$ system.

Textural analysis
Textural analysis of cookies was conducted by using a TA.XTPlus Texture Analyzer (Stable Micro Systems, England, UK), equipped with 3-point bending rig (HDP/3PB), and a 5 kg load cell. Texture analyzer settings were: mode – measure force in compression; pre-test speed – 1.0 mm/s; test speed – 3.0 mm/s; post-test speed – 10.0 mm/s; distance – 5.0 mm; trigger force – 50 g. Ten measurements per each cookies type were made.

Statistical analysis
Results were expressed as the mean of replications ± SD for all measurements. Analysis of variance (ANOVA) and Duncan's multiple range test were used to compare means at 5% significance level by using the statistical data analysis software system STATISTICA (StatSoft, Inc. (2008), version 10.0 (www.statsoft.com).

RESULTS AND DISCUSSION

Data on the physical characteristics of cookies are presented in Table 1. There were significant differences in the thickness of cookies. The thickness was significantly increased by the addition of medicinal plant mixture, at all levels compared to the control sample. The width of cookies was reduced significantly except in the case of cookie enriched with 2% of the plant mixture.

Table 1. Pysical characteristics of cookies

Sample	Width (W) (mm)	Thickness (T) (mm)	Spread ratio (W/T)
Control (K)	53.75 ± 1.08^{b}	9.34 ± 0.79^{b}	5.75
2%	51.93 ± 0.79^{a}	7.99 ± 0.41^{a}	6.50
4%	52.84 ± 3.12^{ab}	7.70 ± 0.24^{a}	6.86
6%	52.39 ± 0.58^{ab}	8.92 ± 0.22^{c}	5.87

Values are means ± SD of ten measurements.
Values with the different superscript within a column are statistically different (P < 0.05).

The cookies exhibited a decreased spread ratio, which ranged from 6.86 (cookie with 4% enrichment) to 5.75 (control sample). Previous studies indicated that two factors affect the spread ratio: expansion of dough by leavening and gravitational flow (Gurjal et al. 2003). Dough flow mostly depends on its viscosity: the higher the viscosity the lower the spread rate (Filipčev et al., 2011). Fuhr (1962) suggested that any ingredient that absorbs water during dough mixing will decrease the spread ratio. According to Jacob and Leelavathi (2007), the type of fat does not make a difference in spread value in the case of cookies made of margarine and bakery fat. On the other hand, cookie dough containing the hydrogenated fat had significantly less spread ratio (Jacob and Leelavathi, 2007). Finney et al., (1950) and later Abboud et al., (1985) concluded that fat type is not an important variable for cookie spread. On the basis of the above mentioned facts, it could be concluded that the addition of 'Vitalplant' in the form of powder, with more coarse particles held less water in comparison to the fine particles of wheat flour (Filipčev et al., 2011), which contributed to larger extend of cookies' spreading (Table 1, Figure 1).

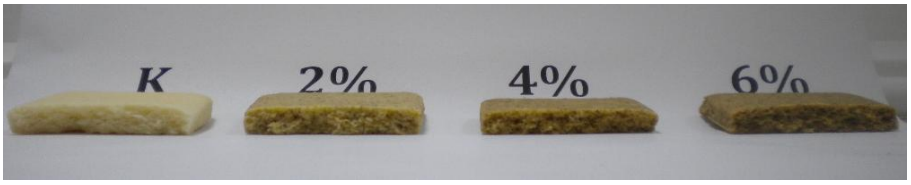

Figure 1. Cross-section of cookies

Sensory evaluation

Sensory quality of produced cookies was defined on the basis of evaluation of appearance, texture, odour and taste. The obtained results are shown in Table 2. Quality category was determined in dependence on scores: unacceptable (<2.5), good (2.5 - 3.5), very good (3.5 - 4.5) and excellent (>4.5).

Table 2. Sensory evaluation of cookies

Attributes	Control	2%	4%	6%
Appearance (surface, shape)	4.25 ± 0.62^{a}	4.50 ± 0.52^{a}	4.42 ± 0.51^{a}	4.55 ± 0.52^{a}
Structure and break	4.17 ± 0.83^{a}	4.33 ± 0.49^{a}	4.50 ± 0.67^{a}	3.73 ± 1.35^{a}
Chewiness	4.33 ± 0.78^{ab}	4.75 ± 0.62^{b}	4.33 ± 0.98^{ab}	3.91 ± 1.04^{a}
Odour	2.67 ± 0.89^{a}	3.92 ± 0.90^{b}	4.33 ± 0.65^{b}	4.55 ± 0.69^{b}
Taste	3.25 ± 0.97^{a}	4.25 ± 0.62^{b}	4.67 ± 0.49^{b}	4.64 ± 0.50^{b}
Weighted mean value	**3.76**	**4.37**	**4.47**	**3.92**

Values are means ± SD of six panellists. Values with the different superscript within a raw are statistically different (P < 0.05). Scores: 1 - unacceptable, 2 - acceptable, 3 - good, 4 - very good, 5 - excellent

Sensory evaluation of the representative attributes of cookies, done by the points-based method showed that the cookie enriched with 4% of the medicinal plant mixture had almost excellent overall quality (Table 2). Scores for individual sensory attributes, except for odour and taste of control sample, indicated very good or excellent quality of all cookies. In addition, data analysis of the odour and taste scores revealed a statistically significant difference between the control sample and all enriched cookies, while no significant differences between the samples were found for the appearance, structure and fracture. The addition of 2% of the medicinal plant resulted in significantly softer product, which was rated the highest for chewiness. Also, 'Vitalplant' mixture applied at three levels of supplementation significantly improved the odour and the taste of the cookies. In general, referring to the results of sensory quality of the cookies, 'Vitalplant' mixture in the form of powder may be used in cookie formulations without producing a negative impact on the sensory attributes.

Colour determinations

The surface colour of a baked product is, together with texture and taste, a very important attribute for the initial acceptability of baked goods by consumers (Zucco et al., 2011). The colour of the cookies' top surface was generated in the baking process possibly due to non-enzymatic browning (Maillard reactions) in the reaction between reducing sugars and amino acids, but also possibly as a results of starch dextrinisation and sugar caramelisation (Chevallier et al., 2000). CIE Lab colour values were determined for cookie samples and presented in Table 3.

Table 3. Colour values of cookie samples

Sample	L*	a*	b*
Top surface			
Control	80.07 ± 0.47^{d}	-1.22 ± 0.44^{a}	25.64 ± 1.16^{a}
2%	65.59 ± 0.76^{c}	1.28 ± 0.64^{b}	29.90 ± 0.75^{b}
4%	57.98 ± 0.88^{b}	2.55 ± 0.45^{c}	31.56 ± 0.91^{c}
6%	54.36 ± 0.78^{a}	2.89 ± 0.33^{d}	30.89 ± 0.69^{d}
Bottom surface			
Control	74.92 ± 1.58^{d}	1.86 ± 1.60^{a}	30.32 ± 2.59^{a}
2%	62.39 ± 1.40^{c}	3.02 ± 1.17^{b}	32.19 ± 1.16^{b}
4%	56.19 ± 0.90^{b}	3.54 ± 0.65^{b}	33.39 ± 0.64^{c}
6%	53.24 ± 0.84^{a}	3.44 ± 0.51^{b}	32.86 ± 0.76^{bc}

Values are means ± SD of twenty-five measurements.
Values with the different superscript within a column are statistically different (P < 0.05)

The analysis of *L** (top and bottom surface) (Table 3) showed that lightness decreased significantly ($P < 0.05$) with the increase in the level of supplementation of the cookies. The obtained results are in accordance with previously published results of the other authors (McWatters et al., 2003; Singh and Mohamed, 2007; Sudha et al., 2007), who found that the incorporation of different composite ingredients may contribute to darker top surfaces (Table 3). Since 'Vitalplant' mixture contained green herbal drugs, significant increase ($P < 0.05$) in red tonality (positive *a** values) was not expected in the case of the both cookie surface colours. However, increase in the level of supplementation was followed by the increase in red tonality. Also, yellow tonality (positive *b** values) significantly ($P < 0.05$) increased with the increase in the mass portion of the medicinal plant mixture in the cookies. Finally, "Vitalplant" mixture addition significantly changed their colour. The acceptability of obtained cookies yellow tonality should be evaluated by consumers.

Textural analysis

Textural properties of cookies were analyzed applying 3-point bending rig which allows measurement of the hardness and resistance of cookies to bend or snap. The hardness refers to the maximum force needed for cookies' fracture, while the fracturability relates to the resistance of cookies to bend. According to many authors (Bourne, 2002; Sudha, 2007; Wekwete and Navder, 2008; Mamat et al., 2010) among the most important factors influencing the mechanical properties of hardness are shape, size and type and amount of fat in the formulations. The measurements showed that cookies containing 4% of the medicinal plant mixture were significantly softer in comparison with other investigated cookies (Table 4).

Table 4. Textural properties of cookies

Property	Control	2%	4%	6%
Hardness, g	1630.7 ± 395.42^{b}	1442.6 ± 249.73^{b}	1198.5 ± 186.63^{a}	1670.4 ± 136.01^{b}
Fracturability, mm	83.02 ± 0.67^{c}	82.44 ± 0.30^{b}	82.05 ± 0.21^{a}	82.95 ± 0.24^{c}

Values are means ± SD of ten measurements.
Values with the different superscript within a raw are statistically different ($P < 0.05$)

On the other hand, hardness of the cookies supplemented at 2 and 6% level was not significantly different between them and the control sample. Since wheat cookies were control ones, and were prepared with the same amount of fat, difference in hardness could have been addressed to the difference in size (width and thickness) as well as spread ratio. Cookie's fracturability, reported as a distance at the point of break is the resistance of cookies to bend, expressed in mm (Table 4). Smaller distance denotes higher fracturability. The obtained results of fracturability indicated statistically important differences ($P < 0.05$) between all investigated cookies. The most fracturable cookie was the sample with the addition of 4% medicinal plant mixture while the control sample was the least fracturable. The obtained results may support above mentioned observation.

CONCLUSIONS

Development of new products by adding 'Vitalplant' mixture in the form of powder to carrier foods such as cookies may provide new market opportunities for bakery producers. Sensory evaluation, colour and texture measurements were found to be sensitive as quality attributes. The addition of the medicinal plant mixture may be used in the cookie formulations without adversely affecting their sensory attributes. Cookies supplemented at 4% of the medicinal plant mixture were estimated by sensory evaluation and instrumental measurements to be the best. Further research needs to be done in order to examine consumers' acceptance of the new products, especially in terms of colour.

Based on their overall good sensory quality, and previously confirmed antioxidant and antimicrobial properties of the mixture, new cookie formulations can broaden the utilization of this mixture and may be regarded as health-promoting functional foods.

ACKNOWLEDGEMENTS

This paper is a result of the researches within the Project No. TR 31029 (The Ministry of Education and Science, Republic of Serbia) and the Project No. 114-451-3926/2011-03 (The Provincial Secretariat for Science and Technological Development, Province of Vojvodina, Republic of Serbia).

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ACCEPTANCE OF FOOD ADDITIVES IN HUNGARY AND SPAIN: AN EXPERIMENTAL STUDY

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ABSTRACT: The level of worry against food additives is higher in Hungary (81%), than in Spain (54%) (Eurobarometer, 2010), probably as a result of different level of knowledge and attitudes.
Results of a previous survey carried out by the authors in Hungary and Spain ($N_{Hungary}$= 437, N_{Spain}= 348) showed a strong demand for artificial additive-free products in both countries, even at higher prices. Thus, the aim of our work was to analyse and compare the willingness to buy (WTB) and preference of foodstuffs containing natural and artificial food additives in Hungary and Spain.
For this purpose a conjoint analysis was done. Conjoint cards were created from two groups of food additives (preservatives and packaging gases), and from two "model foodstuffs". One foodstuff expected high additive content (chips) and one expected low additive content (pre-packed sliced cheese). These foodstuffs were used to stimulate shopping situation. Three factors were selected: "preservatives" (artificial/natural), "packaging gases" (contains/does not contain) and "price" (average+10%/average+20%). For the conjoint analysis six cards were selected. Results were collected via Internet.
On the basis of the conjoint analysis the presence of "natural preservatives" was the most important factor in case of cheese in both countries. Regarding chips the "natural preservatives" were the most influencing factor in Hungary, while the presence of "packaging gases" in Spain. With the help of cluster analysis 3 clusters were created in each country. Respondents were sensitive to the "prices" of the foodstuffs in both countries, so it must be taken into consideration during product development and the production of new foodstuffs.
Key words: *food additives, conjoint analysis, preservatives, packaging gases, willingness to buy*

INTRODUCTION

Nowadays consumers expect the foodstuffs they buy and eat to be safe and convenient as well as tasty, pleasant to eat and healthy. These exceptions require ingredients to be used improving the appearance of foodstuffs and increase their health benefits. In order to fulfil these consumer demands, the food industry uses different food additives.
According to the Eurobarometer survey of 2005 when concerns about a range of listed risks/problems associated with food were analysed, the rate of worry about "additives like colours, preservatives or flavourings used in food or drinks" in Hungary was really high (76%), while in Spain lower (46%) (EU mean: 57%) (Eurobarometer, 2006). Similar results were detected in 2010, when 81% of the Hungarian participants worried, while in Spain this proportion was lower (54%) (EU mean: 66%) (Eurobarometer, 2010). Based on our survey conducted in 2011, Hungarian respondents judged "food additives" to be more hazardous (mean: 3.15; on a 1-5 Likert scale, where 1: not hazardous at all – 5: really hazardous; N= 437) – although not significantly – than Spanish participants (mean: 3.08; N= 348). In the just now mentioned survey a strong demand was expressed for additive-free foodstuffs in both countries. In Hungary 80.1% of the participants, in Spain 73.3% would buy foodstuffs without food additives, even if they had different colour or consistence than it used to. Furthermore, these consumers were ready to pay extra money for these products, 10% (in Hungary 15.1% of the respondents and 20.7% in Spain) or 20% more frequently (in Hungary 21.7% of the respondents and 10.9% in Spain). Thus, the aim of our work was to analyse and compare

the WTB and preference of foodstuffs containing natural and artificial food additives in Hungary and Spain.

MATERIAL AND METHODS

According to the authors' previous survey Hungarian respondents judged "preservatives" ($p \leq 0.001$) to be more hazardous than Spanish people, while in case of "packaging gases" the opposite tendency was observed ($p \leq 0.001$). Furthermore, unambiguous consumer demand was found for foodstuffs free of food additives even at a higher price in both countries. The most frequently mentioned acceptable extra rates in case of common products (e.g. bread, cold cuts) were 10% and 20%. Thus for the conjoint study three factors were chosen: "preservatives" (artificial/natural), "packaging gases" (contains/does not contain) and "price" (average+10%/average+20%). Average prices were determined on the basis of market data. On the basis of the selected factors conjoint cards of two "model foodstuffs" were created and used to simulate a shopping situation. One foodstuff expected high additive content (chips) and one expected low additive content (pre-packed sliced cheese) (Figure 1). In order to avoid the effect of the order of the selected foodstuffs two questionnaires were designed in both countries (one started with cheese and continued with chips, and the other in reversed direction). For the conjoint study six cards were chosen, which were set out in a fix order (Table 1). The selected foodstuffs' consumption frequency was asked in the conjoint questionnaire. Results were collected via Internet with the help of an e-mail list – compiled from previous studies – furthermore participants were asked to forward the link if they could. The data collection period was between autumn 2011 and winter 2012. 250 questionnaires were collected in Hungary, and 211 in Spain, but not all of them were suitable for the conjoint analysis (Hungary: 167, Spain: 147). Data were analysed with the help of SPSS 17.0 statistical software (conjoint analysis, K-means cluster analysis and Chi^2 test).

Table 1. The applied conjoint cards in order

	Preservatives		Packaging gases		Price	
	artificial	natural	contains	does not contain	+10%	+20%
1		X		X	X	
2		X		X		X
3	X			X	X	
4		X	X		X	
5	X			X		X
6		X	X			X

Figure 1. Samples from the conjoint cards (Spain)

RESULTS AND DISCUSSION

Utilities and relative importance of the analyzed factors

According to the Spanish results small difference was found between the utility of "artificial/natural preservatives" and the "presence/absence of packaging gases" regarding cheese. However, in both countries the most preferred aspect was the presence of "natural preservatives". In case of "packaging gases", higher relative importance rates were perceived in Spain, than in Hungary. In case of Hungary the utilization of "packaging gases"

was rejected in chips and certain indifference was observed regarding cheese. Increasing "price" had negative influence on the utility. In case of cheese the "price" was more important for Hungarian respondents, than for Spanish ones (Table 2).

Table 2. Relative importance of each factor and utility for each level (Cheese)

Factor	Level	Utility		Relative importance (%)	
		Hungary	Spain	Hungary	Spain
Preservatives	**Natural**	0.675	0.595		
	Artificial	-0.675	-0.595	42.765	40.696
Packaging gases	**Presence**	-0.076	0.588		
	Absence	0.076	-0.588	26.421	39.836
Price	**+10%**	-0.607	-0.388		
	+20%	-1.214	-0.776	30.814	19.468

Analyzing the results regarding the chips it was found that one of the most important factors of rejection was the presence of "artificial preservatives" in both countries. "Packaging gases" attracted higher interest in Spain. Presence of "packaging gases" was a less important factor for Hungarian participants; however Spanish respondents preferred the utilization of these substances mainly in case of chips. The WTB is decreasing with the increasing of the "price" in both countries (Table 3).

Table 3. Relative importance of each factor and utilities for each level (Chips)

Factor	Level	Utility		Relative importance (%)	
		Hungary	Spain	Hungary	Spain
Preservatives	**Natural**	0.964	0.578		
	Artificial	-0.964	-0.578	**55.984**	40.533
Packaging gases	**Presence**	-0.174	0.619		
	Absence	0.174	-0.619	25.197	**41.251**
Price	**+10%**	-0.210	-0.293		
	+20%	-0,419	-0.585	18.819	18.216

Cluster analysis

In order to explore differences amongst the consumers' decisions and to determine similar consumer groups, on the basis of the utilities, a K-means cluster analysis was performed, and 3-3 clusters were created in both countries (Table 4).

Hungary

Informed price sensitive: Members of this cluster prefer cheese and chips with "natural preservatives" and without "packaging gases", but they do not want to pay extra money for these foodstuffs. Thus these consumers are price sensitive.

Inert: Most of the Hungarian respondents belong to this cluster. These consumers prefer "natural preservatives", and they do not care about the presence of "packaging gases". They are price sensitive.

Aspire to health: Participants of this cluster show reference marks to health, but at the same time they want to enjoy the consumed foodstuffs. They prefer chips with "natural preservatives" and without "packaging gases", but they do not want to pay too much extra money for these foodstuffs.

The presence of "natural preservatives" is the most important factor for the "informed price sensitive" cluster regarding pre-packed sliced cheese, while in case of chips for the "aspire to health" cluster (Table 4).

Spain

Lover of natural: The most important factor for this cluster is the use of "natural preservatives" instead of artificial ones. Nevertheless members of this cluster do not take

care about the presence of “packaging gases”, furthermore they do not want to pay more money for these foodstuffs.
Convenient: Participants of this cluster like pre-packed and easy-to-handle foodstuffs like the pre-packed cheese and chips. They prefer foodstuffs with “natural preservatives”, but practicality is more important for them. At the same time they are moderately price sensitive, they do not want to pay extra money for these foodstuffs.
Inert: Most of the Spanish participants belong to this cluster. They prefer cheese and chips with “natural preservatives” and with “packaging gases”, however they do not want to pay extra money for these products. This cluster is the most price sensitive.
Presence of “packaging gases” got positive acceptance by the members of the clusters, mainly by the “convenient” cluster. “Natural preservatives” got also favourable judgement, and this was the most important thing for the “lover of natural” cluster in case of both foodstuffs (Table 4).

Table 4. Cluster utilities of each level

Foodstuff	Factors		Clusters					
			Hungary				Spain	
			Informed price sensitive (N= 58)	Inert (N= 68)	Aspire to health (N= 41)	Lover of natural (N= 38)	Convenient (N= 34)	Inert (N= 75)
Cheese	Preservatives	Natural	1.09	0.28	0.75	1.61	0.18	0.27
		Artificial	-1.09	-0.28	-0.75	-1.61	-0.18	-0.27
	Packaging gases	Presence	-0.53	0.33	-0.11	0.07	1.94	0.24
		Absence	0.53	-0.33	0.11	-0.07	-1.94	-0.24
	Price		-0.98	-0.05	-1.00	-0.12	-0.24	-0.59
Chips	Preservatives	Natural	0.69	0.58	1.98	1.34	0.21	0.36
		Artificial	-0.69	-0.58	-1.98	-1.34	-0.21	-0.36
	Packaging gases	Presence	-0.28	0.16	-0.58	0.29	1.98	0.17
		Absence	0.28	-0.16	0.58	-0.29	-1.98	-0.17
	Price		-0.47	-0.16	0.08	-0.10	-0.20	-0.44

Cheese and chips consumption in the clusters

Hungary

Only 37.8% of the Hungarian participants consume pre-packed sliced cheese “1-3 times per month” or more often. Members of the “informed price sensitive” cluster consume pre-packed cheese the most frequently (39.6% at least “1-3 times per month”), closely followed by the “aspire to health” cluster (39.1% at least “1-3 times per month”) (Figure 2).
35.9% of the participants consume chips “1-3 times per month” or more often, and 59.3% “less than once per month”. Like in case of cheese, members of the “informed price sensitive” cluster consume chips the most frequently (44.8% at least “1-3 times per month”), closely followed by the “aspire to health” cluster (43.9% at least “1-3 times per month”) (Figure 3).

Spain

Spanish participants consume pre-packed sliced cheese (83.7% “1-3 times per month” or more often) and chips (81.7% “1-3 times per month” or more often) more frequently than Hungarians (Figure 2, 3).
Regarding cheese, amongst the members of the “once a week or more often” consumers the “lover of natural” cluster have the highest rate (73.7%), while in sum the members of the “inert” cluster consume it the most frequently (86.6% at least “1-3 times per month”) (Figure 2).
Consumers of the “lover of natural” cluster have the highest rate amongst the “once a week or more often” chips consumers (42.1%), but the members of the “inert” cluster are the highest chips consumers (89.4% at least “1-3 times per month”) (Figure 3).

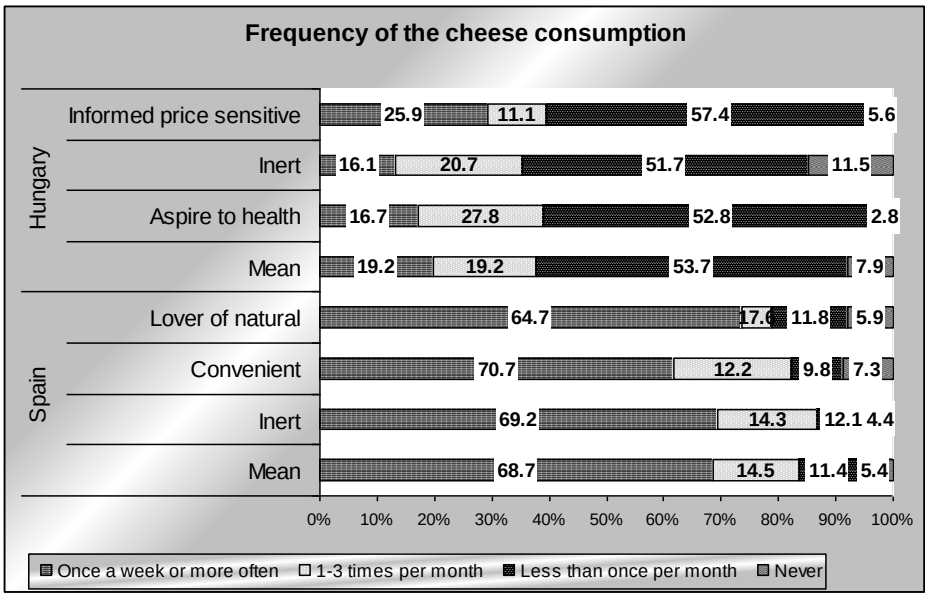

Figure 2. Cheese consumption of the three clusters in Hungary and Spain

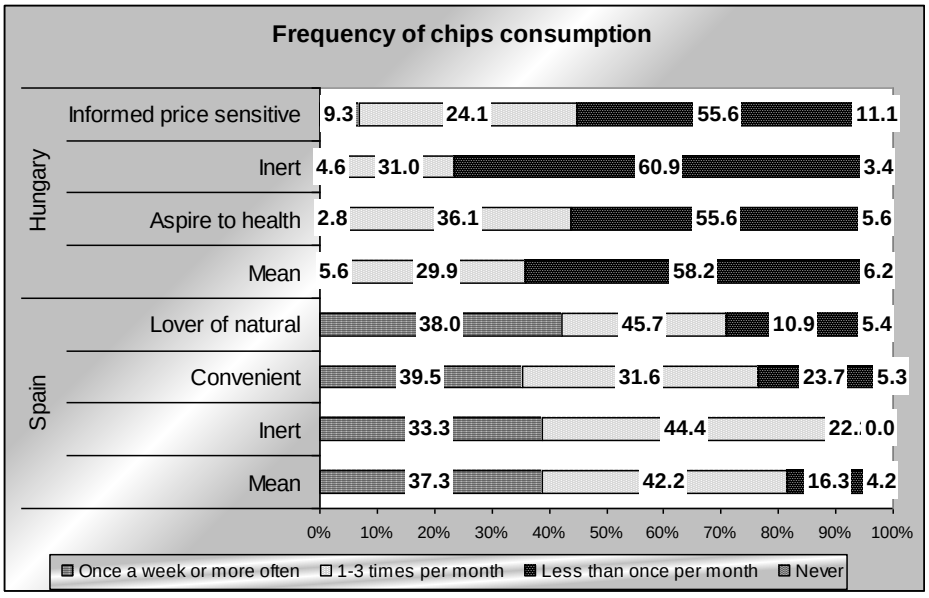

Figure 3. Chips consumption of the three clusters in Hungary and Spain

CONCLUSIONS

According to the results of the cluster analysis in case of pre-packed sliced cheese the most important factor influencing the preferences was the presence of “natural preservative” in both countries. The presence of “natural preservatives” was the most influencing factor regarding chips in Hungary, while the presence of “packaging gases” in Spain. Results of a previous study carried out by the authors were not confirmed by the findings of this conjoint analysis, since strong rejection against the presence of “packaging gases” was not observed in Spain. Spanish consumers probably agree with the utilization of gases in these foodstuffs, thus they become easier to handle and more practical. By means of a cluster analysis three clusters was designed in each country which had different characteristics. Most of the participants belong to the “inert” cluster in both countries – the most frequent cheese and chips consumers in Spain – where the members prefer “natural preservatives”, do not care about “packaging gases”, and they are price sensitive. Members of the “aspire to health”

cluster – which were frequent cheese and chips consumers – can be influenced by new information and trends in Hungary and in case of Spain participants of the “lover of natural” cluster. Both countries’ respondents showed price sensitive attitude so it must be taken into consideration during the development and production of new foodstuffs.

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KNOWLEDGE AND JUDGEMENT OF FOOD ADDITIVES IN THREE EUROPEAN COUNTRIES

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ABSTRACT: Due to the rushing lifestyle and the constantly rising demands for convenience food, additives became important ingredients of foodstuffs.
According to the results of the Eurobarometer survey (2010) there were differences between the reported level of worry against food additives in Hungary, Spain and Romania. In order to get more information about the main reasons for these differences a self administered questionnaire was developed in 2009 in CFRI to evaluate Hungarian consumers' knowledge and judgement about food additives. In the frame of a "pilot study" 400 respondents' data were collected. Following the analysis of the data a concise and more targeted questionnaire was designed, which was translated into Spanish and Romanian. This questionnaire was filled out in these three countries via Internet.
On the basis of the results, differences were found in the judgement and knowledge of food additives amongst the countries, and on the basis of the socio-demographic factors, too. These differences can be caused by consumers' mixed and ambiguous knowledge, furthermore by the countries' different communication about these ingredients.
Key words: *food additives, knowledge, risk perception, questionnaire*

INTRODUCTION

Thanks to the continually changing lifestyle and consumption habits, utilization of food additives became widespread and common. According to the Eurobarometer "Risk issues" survey conducted in 2005, 7% of the respondents mentioned "food additives" as a risk associated with food in an open-ended question (where response alternatives were not presented) (Eurobarometer, 2006). In the "Food-related risks" survey in 2010 this rate was a little bit higher, 9% (Eurobarometer, 2010). In the above mentioned surveys when people's concerns about a range of named risks/problems associated with food were analysed (prompted responses) against "additives like colours, preservatives or flavourings used in food or drinks" higher rates of worry were found (57% in 2005 and 66% in 2010).
In Hungary the prompted response against "additives like colours, preservatives or flavourings used in food or drinks" in both Eurobarometer surveys were very high, 76% in 2005, and 81% in 2010. In Spain these rates were much lower, 46% in 2005, and 54% in 2010. Romania participated only in the last Eurobarometer survey (2010). According to the Romanian respondents' results the rate of concern about food additives was high 74%.
On the basis of the results of the mentioned surveys differences were found in the rate of concerns against food additives in Hungary, Spain and Romania.
The aim of our study was to try to collect more detailed information in order to gain knowledge about these differences.

MATERIAL AND METHODS

A self-administered questionnaire was developed to evaluate the knowledge and judgement of Hungarian consumers' about food additives, from which in the frame of a "pilot study" 400 respondents' data were collected. Following the analysis of the data a concise and more

targeted questionnaire was designed, which was translated into Spanish and Romanian, too. Participants in the questionnaire were asked to judge different risk factors on a 1–5 Likert scale (1: not hazardous at all, 5: really hazardous), questions related to knowledge "Do you know the meaning of food additives used by food industry?" and "Do you know what "E-numbers" mean on food packaging?". Respondents' detailed knowledge was analysed with the help of six statements. Some socio-demographic data were asked, too. This questionnaire was filled out in Spain on paper (with the assistance of a nearly representative sample, N= 200) and via Internet. In Hungary and in Romania the questionnaire was filled out only through Internet, which is a comfortable and cost effective too however the sample is restricted to Internet users, who as a group may not be representative of the population as a whole (Fox et al., 2003). Altogether 437 fully completed questionnaires were collected in Hungary, 348 in Spain (200 paper and 148 internet based) and 386 in Romania. Results were collected in 2011 (Hungary: spring-summer, Spain: summer-autumn and Romania: autumn-winter). Data were analysed by means of SPSS 17.00 statistical software.

Distribution of socio-demographic factors

Female respondents were in majority in the examined countries. In Hungary and Romania the distribution of respondents' age was similar; however in Spain it was different – partly due to the nearly representative 200 consumers' sample. Most of the participants were from big cities in the three countries, and less from villages or other settlements. Because of the different education systems equal groups can be formed only in case of Hungary and Romania (Table 1).

Table 1. Distribution of socio-demographic factors in the analysed samples of the three countries

		Hungary	Spain	Romania
Gender	Female	69.3%	59.5%	75.6%
	Male	30.7%	40.5%	24.4%
Age	18–24 years	30.4%	7.2%	47.7%
	24–44 years	52.9%	48.3%	40.9%
	Over 45 years	16.7%	44.5%	11.4%
Place of residence	City with more than 20.000 inhabitants	71.4%	74.1%	75.9%
	City with less than 20.000 inhabitants	13.3%	10.3%	13.2%
	Village, other settlements	15.3%	15.5%	10.9%
Highest level of education	Lower than school leaving examination	14.2%	11.5%	15.3%
	School leaving examination		34.5%	
	Bachelor degree	32.0%		66.6%
	Master degree	53.8%	54.0%	18.1%

RESULTS AND DISCUSSION

Risk perception

Based on the polled people in the three countries the most hazardous risk factors were different from food additives. In Spain (4.68) and Romania (4.79) "chemical residues (e.g. pesticides)" ended up in the first place, while in Hungary (4.51) it was the "chemical substances from environmental pollution". Factors considered to be the less hazardous were also different in the countries. In Hungary "gases of the modified atmosphere" (2.65), in Spain "other food additives (e.g. gelling agents)" (2.97) while in Romania "preservatives" (3.46) were the less hazardous factors. Regarding the "gases of the modified atmosphere" a high aversion was detected in Romania (3.77), while significantly ($p \leq 0.001$) less in Spain (3.34) and in Hungary (2.65). Romanian respondents reported significantly higher ($p \leq 0.001$) risk against "food additives", "E-numbers" and "GMO foodstuffs", than participants in Hungary and in Spain. Furthermore it was found that Romanian participants perceived higher risk

against the different groups of food additives listed amongst the risk factors, than respondents in Hungary and Spain.
Significant difference ($p \leq 0.001$) was found between the judgement of "food additives" and "E-numbers" in Spain and Romania, but not in Hungary. This proves that Spanish and Romanian participants were not at all aware of the fact that these two terms cover the same substances.
"Sweeteners" and "preservatives" with the term "artificial" presented a significantly ($p \leq 0.001$) higher level of worry in the three countries' participants than the same factors without the term "artificial".
Results showed that Hungarian participants felt the biggest difference between the listed risk factors, while Romanian respondents the smallest (Table 2).

Table 2. Perceived risk levels in three European countries (1: not hazardous at all, 5: really hazardous)

Risk factors	Hungary (a)	Spain (b)	Romania (c)	SD
Gases of the modified atmosphere	2.65	3.34	3.77	a-b, c; b-c
E-numbers	3.09	3.35	4.23	a-b, c; b-c
Sweeteners	3.13	3.07	3.69	c-a, b
Food additives	3.15	3.08	3.79	c-a, b
Other food additives (e.g. gelling agents)	3.26	2.97	3.67	a-b, c; b-c
Preservatives	3.38	2.97	3.46	b-a, c
Artificial sweeteners	3.43	3.27	4.04	c-a, b
GMO foodstuffs	3.46	3.48	4.29	c-a, b
Flavourings	3.49	3.19	4.00	a-b, c; b-c
Substances migrating from packagings	3.79	4.11	4.03	a-b, c
Artificial preservatives	3.80	3.56	4.13	a-b, c; b-c
Pathogenic microorganisms	4.04	4.50	4.52	a-b, c
Antibiotics and hormones in meat and milk	4.27	4.56	4.31	b-a, c
Pathogenic mould and mycotoxins in foodstuffs	4.37	4.58	4.44	b-a, c
Chemical residues (e.g. pesticides)	4.48	4.68	4.79	a-b, c; b-c
Chemical substances from environmental pollution	4.51	4.67	4.55	b-a, c

Risk perception on the basis of socio-demographic factors

It was difficult to find unambiguous tendency regarding the three countries. Some outstanding observations will be demonstrated hereinafter, with special regard to the judgement of "food additives" and "E-numbers".
Most of the risk factors were judged to be more hazardous by females than by males in Hungary and Romania. Furthermore female participants linked higher level of risk to "food additives" and "E-numbers" than male respondents.
In Hungary and Spain the same trend was found regarding the age groups. In most of the cases younger respondents (18-24 ys) reported lower hazardous level than older ones (over 45 ys). In Romania the opposite was observed. Related to the knowledge of "food additives" and "E-numbers" significant connections ($p \leq 0.05$ and $p \leq 0.001$) were found amongst the age groups only in Hungary. Young Hungarians (18-24 ys) thought these factors less hazardous than older participants (over 45 ys).
Respondents from big cities (over 20.000 habitants) reported higher hazardous level regarding most of the listed risk factors ─ food additives and "E-numbers" too ─ than habitants from smaller settlements (village or other settlements) in Spain and Romania. The opposite tendency was observed in Hungary, where participants from smaller settlements linked higher level of hazard to most of the risk factors.
Judgements of the listed risk factors were different even within one country on the basis of the highest level of education. Differences were detected between the evaluation of the

hazardous levels of “food additives” and “E-numbers”. In Romania respondents with the lowest level of education (school leaving examination or lower educated) reported the lowest risk level against these factors, while in Hungary these participants perceived the highest one ($p \leq 0.001$). Spanish participants with the highest level of education (Master degree) perceived the lowest risk level in case of both factors ($p \leq 0.001$ and $p \leq 0.05$).

Knowledge of food additives

Romanian participants reported the highest knowledge (92.5%) about food additives, while Spanish participants the lowest (84.2%). Regarding “E-numbers” Hungarians (72.5%) and Romanians (70.5%) were self-confident, however Spanish people were more uncertain (45.4%).

Knowledge of food additives on the basis of socio-demographic factors

There were more Hungarian and Romanian females ($p \leq 0.05$) who answered that they knew food additives and “E-numbers” than males. In Spain a similar tendency was not observed.
The level of knowledge of food additives was rising with the age ($p \leq 0.05$) in Romania. Middle aged Spanish respondents (25-44 ys) had the highest level of knowledge ($p \leq 0.05$), however in Hungary this group had the lowest ($p \leq 0.05$).
According to the place of residence significant differences were not observed.
The knowledge of food additives and “E-numbers” was in parallel with the rising of the highest level of education ($p \leq 0.05$, and $p \leq 0.001$) in Spain. Respondents with Bachelor degree had the highest knowledge, and those who had Master degree had the lowest (food additives: $p \leq 0.05$) in Hungary.

Detailed knowledge of food additives

On the basis of the results, Hungarian participants reported the highest knowledge, while Spanish respondents the lowest (Figure 1). Compared the answers given to the previous questions about the knowledge of food additives and “E-numbers” to the answers about one of the statements “Every food additives can be linked to an “E-number”, it can be said that respondents previously overestimated their knowledge in the three countries. Only 27.6% of the Spanish participants knew that “Natural additives also have “E-numbers” and 37.9% that “the role of “E-numbers” is to easily identify food additives”.

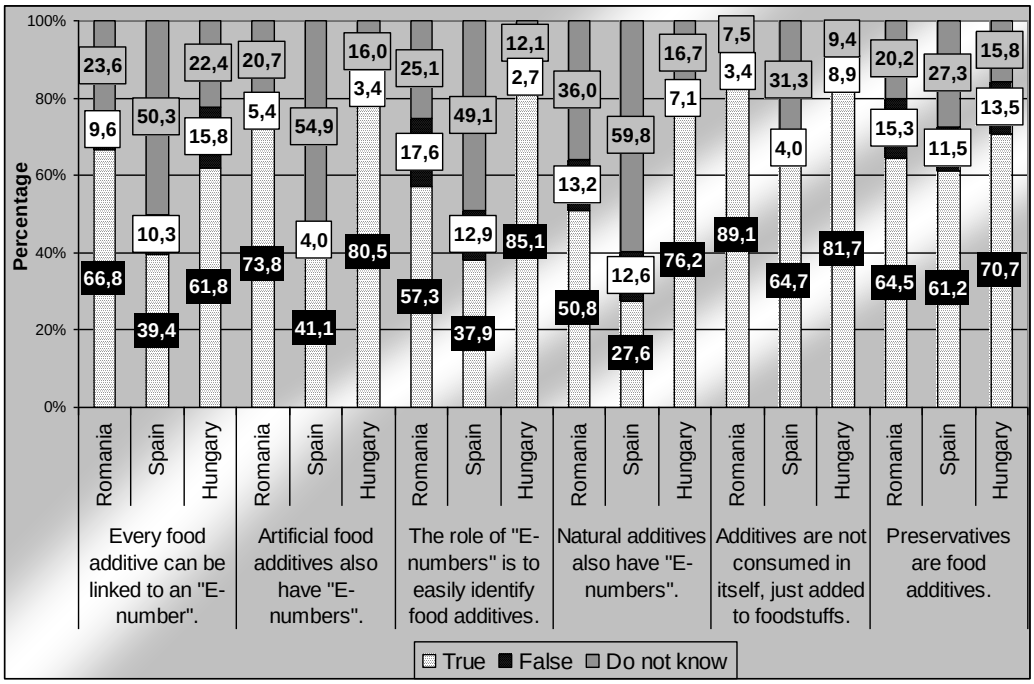

Figure 1. Knowledge of food additives and “E-numbers” on the basis of statements in the three European countries

Detailed knowledge of food additives on the basis of socio-demographic factors

Regarding gender mixed knowledge levels were perceived in Spain and Hungary. Romanian women were a little bit more informed about food additives than men.

According to the age groups in Spain and Romania an explicable tendency was not observed. The youngest group (18-24 ys) had better knowledge about food additives – those who previously linked the lowest risk levels to most of the listed risk factors – than the oldest ones (over 45 ys) in Hungary.

Spanish respondents from small cities (city with less than 20.000 inhabitants) had the less detailed knowledge about food additives, while in Romania – except one statement "the role of "E-numbers" is to easily identify food additives" which was significantly ($p \leq 0.05$) less known by participants from cities with less than 20.000 inhabitants, than others – and Hungary inhabitants of villages or other settlements had the lowest.

In case of some statements the level of knowledge was rising in parallel with the level of education, but it was not overall in Spain. Hungarian participants with Bachelor degree had the best detailed knowledge of the statements. Occasionally respondents with Bachelor degree had the highest knowledge level, while in all cases respondents with Master degree had the lowest in Romania. It is important to note that more Hungarian and Romanian consumers had higher education level than Spanish ones.

CONCLUSIONS

On the basis of a self-administered questionnaire it was found that according to the Hungarian, Spanish and Romanian participants the most hazardous factors were independent from food additives. The leaders of the hazard list were the "chemical substances from environmental pollution" and the "chemical residues (e.g. pesticides)". Romanian respondents reported higher level of hazard to factors in connection to food additives, but – together with the Spanish participants – thought "E-numbers" to be significantly more hazardous than "food additives". Thus they did not know the exact connection between these two terms. Hungarian participants made the biggest difference during the judgement amongst the listed factors, while Romanian participants the least.

It was difficult to determine unified tendencies in the three examinded countries on the basis of the socio-demographic factors. It can be highlighted that females perceived higher risk against the different risk factors, and Hungarian and Romanian females had better knowledge (not detailed) of food additives and "E-numbers". Hungarian and Spanish young respondents (18-24 ys) linked lower hazardous level to most of the listed risk factors. Furthermore in Hungary this group had the most detailed knowledge about food additives. Inhabitants from smaller Spanish and Romanian settlements reported lower level of hazard against the listed risk factors, and Romanian participants had the lowest detailed knowledge about food additives. The highest educated (Master degree) Spanish participants linked the lowest hazardous level to food additives and "E-numbers" as risk factors, furthermore these people reported the better knowledge of both factors, and the most detailed knowledge too.

On one hand differences in the judgement of food additives can be due to consumes' mixed and ambiguous knowledge, and on the other hand to the different types of communication about these ingredients. Food additives are one of the most well-known food ingredients in the Hungarian media, while in Spain and Romania this is not typical at all.

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CONSUMER ATTITUDES TO BROILER MEAT AND PRODUCTS

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ABSTRACT: The aim of this paper was to select the most acceptable housing system of broiler meat production evaluating consumers attitudes and to investigate quality of raw type sausages made of chicken meat, applying quantitative descriptive sensory analysis.
Based on the survey conducted among consumers of different sex, age and education, it was concluded that majority of consumers of both sexes finds that the price of broiler meat produced in free range production is justifiably higher because of the quality of meat provided. Meat should be labelled according to production system and quality of meat should be controlled by scientific institution according to the opinion of female consumers, whereas male consumers stated that meat quality should be controlled by producers and inspection. Compared sausages samples are made with different chicken meat type: A – commercial broiler, B – commercial broiler and Naked Neck chickens (50:50 ratios) and C – Naked Neck chicken. In all three variants of sausages 20% of pork fat was added. Sensory evaluation was done after roasting. System of 9 points was used for sausages quality attributes (parameters) scoring: 1-exceptionally unacceptable to 9-exceptionally acceptable. Following attributes were evaluated/scored: external appearance, cross-section appearance, colour, smell, taste and texture. Thirty untrained panellists participated in scoring. The appearance of all examined sausage was very good. The colour of group B and C were evaluated as acceptable, but not the usual for this sausage type. Sausages from group A had lighter colour than expected and assessed as too pale. Group B had the highest scores for smell and taste. As a result of conducted sensory analysis, sausages from variant B were selected as the best of compared samples, with overall scores of 7.87.
Key words: *Broiler meat, product, consumer, attitudes*

INTRODUCTION

It is general knowledge that in each production chain the most important is the last link – consumer. For every production it is very important to know why the consumers are purchasing the product and what are their preferences in that regard. In countries with developed poultry production, first and few studies of the consumer attitude towards poultry products appeared in the sixties. However, in the eighties, these studies have become more prominent and intensive, so, today, topics dealing with consumer attitudes have the most prominent place on all poultry meetings.
Favourable circumstance is that in our country, considerable attention was paid on studies of the consumer attitudes towards poultry products. From seventies to late eighties, there were several studies focused on different directions. The questionnaire poll was used as investigation method, which included predominantly consumers from Belgrade as the largest market of poultry products in our country (Mašić and Pavlovski, 1984; Mašić and Pavlovski, 1991; Pavlovski, 1981a; Pavlovski, 1981b; Pavlovski, 1982; Pavlovski and Mašić,1993; Pavlovski et al., 1980; Pavlovski and Mašić,1994; Pavlovski et al., 2002). Studies of the consumer attitudes towards poultry meat originating from extensive rearing system in our country were carried out by Rodić et al., 2003.
The aim of this paper was to select the most acceptable housing system of broiler meat production evaluating consumers' attitudes and to investigate quality of sausages made of chicken meat, applying quantitative descriptive sensory analysis.

MATERIAL AND METHODS

Study of the consumers attitudes towards poultry meat coming from free range system of production comparing to the commercial broiler included survey of consumers of different sex (100 female and 100 male), ages 21-64 years, and level of education (high school-HSE and faculty-FE .Survey was carried out on the territory of the city of Belgrade on a simple random sample of 200 respondents using a questionnaire. Data were analized by SPSS 15.0.
The aim of the second part of investigation was to select the most acceptable raw type sausage made of chicken meat (from free range system), applying quantitative descriptive sensory analysis. Compared sausages samples are made with different chicken meat type: A – commercial broiler, B – commercial broiler and Naked Neck chickens (50:50 ratios) and C – Naked Neck chicken. In all three variants of sausages 20% of pork fat was added. Sensory evaluation was done after roasting. System of 9 points was used for scoring: 1-exceptionally unacceptable to 9-exceptionally acceptable. Acceptability of following attributes was valuated/scored: external appearance, cross-section appearance, colour, smell, taste and texture. Thirty untrained panellists participated in scoring .Data bases were analyzed using software program SPSS 15.0 All significant differences established based on variance analysis were evaluated using T test.

RESULTS AND DISCUSSION

In regard to the question “How important in the process of production are the following: welfare, environment, profit? (offered answers were: very important, important, not important, no opinion) “, male respondents answered that the environment were very important (76.9%), also animal welfare (46.1%), whereas 46.2% answered that profit was important. Respondents of female sex found animal welfare to be important (62.5%) and environment very important (50.0%) and important (41.5%), whereas the profit was on second place with score (54.2%). Based on analyzed answers of male and female respondents, it could be concluded that the influence of sex/genus, age and level of education on what could not be established.
In regard to the question “Which type of poultry meat, coming from which system (floor system with free range, organic production) should be the most expensive or the cheapest from conventional system or floor system without range?“ Respondents of different sex, age and level of education answered in following way: 100% of male respondents and 83.4% females, age from 21-35 years (100%) and high education level (92.4%) thought that the meat produced in the organic system should be the most expensive. Interesting is that lot of respondents of both sexes (46.2 % males and 29.2% females) from 51-64 years of age, 50% and 45,4% of respondents of medium education level, had no opinion about the production system from which the poultry meat should be the cheapest.

Table1. Question "Should the meat on the market have the indication of the system of origin?" and answers grouped according to the sex, age and education level

Question/Answer		**Sex**		**Age**			**Education level**	
		M	**F**	**21-35**	**36-50**	**51-64**	**HSE**	**FE**
Should the meat on the market have indication of the rearing system	yes (%)	92.3	83.3	80.0	86.7	87.5	81.8	88.5
	no (%)	7.7	16.7	20.0	13.3	12.5	18.2	11.5

Table 2. Question " Who should issue the certificate of origin of meat?" and answers grouped according to the sex, age and education level

Question/Answer		Sex		Age			Education level	
		M	F	21-35	36-50	51-64	HSE	FE
Who should issue the certificate of origin of meat	Producers	7.7	4.2	0	0	12.5	0	7.7
	Producers but with present inspection	46.1	12.5	40.0	33.3	12.5	9.1	30.8
	Government inspection	0	12.5	20.0	6.7	6.2	0	11.5
	Specialized certification firms	23.1	20.8	0	13.3	31.3	27.3	19.2
	Scientific inspections	23.1	50.0	40.0	46.7	37.5	63.6	30.8

From table 1, it is obvious that 92.3% of male and 83.3% of female respondents, 87.5% from 51-64 years of age and 88.5% of respondents of high education (faculty degree) thought that meat placed on the market should have the indication of the system of production .
Table 2 shows anwers to the question: „Who should issue the certificate of origin"? Most of male respondents (46.1%) thought that producers with present isnpection should issue the certificates of origin, whereas 46.7% of female respondents thought that scientific inspections should be responsible for this.

Table 3. Consumer attitudes on meat from free range system

Question/Answer		Sex		Age			Education level	
		M	F	21-35	36-50	51-64	HSE	FE
Is the meat produced in free range system healthier that meat produced in conventional system	yes	61.5	50.0	60.0	26.7	75.0	63.6	50.0
	no	30.8	20.8	20.0	46.6	6.2	18.2	26.9
	no opinion	7.7	29.2	20.0	26.7	18.8	18.2	23.1
Should the price of meat coming from different production systems also be different	yes	69.2	79.2	100	66.7	75.0	54.5	84.6
	no	15.4	8.3	0	20.0	6.2	9.1	11.5
	no opinion	15.4	12.5	0	13.3	18.8	36.4	3.9
Reasons for difference in prices	Meat quality	53.8	50.0	40.0	33.3	68.7	63.6	46.2
	Production costs	30.8	25.0	60.0	26.7	18.7	19.2	30.8
	Quality and costs	15.4	8.3	0	20.0	6.3	9.1	11.5
	No opinion	0	16.7	0	20.0	6.3	9.1	11.5

Consumers prefer poultry meat produced in free range system. In regard to the question: „Is the meat produced in free range system healthier that meat produced in conventional system"?, 61.5% of male and 50.0% of female consumers thought that it was, 60.0% of consumers from 21-35 years of age and 63.6% of consumers with high school education. 69.2% of male and 79.2% of female consumers thought that prices of poultry meat from different production system should differ and this attitude was mostly influenced by the age (21-35) and education level (FE).
Qaulity of meat was the most improtant factor influencing the difference in prices of meat produced in different production systems.
The appearance of all examined sausages was very good. The colour of groups B and C was evaluated as acceptable, but not the usual for this sausage type. Sausages from group A had lighter colour and assessed as too pale. Group B had the highest scores for smell and taste. As a result of conducted sensory analysis, sausages from variant B were selected as the best of compared samples, with overall scores of 7.87.

Table 4. Results of sausages sensory evaluation (mean ± standard deviation)

Sensory characteristics	A	B	C
External appearance	6.59^{a} ± 0.52	7.62^{b} ± 0.47	7.83^{b} ± 0.63
Cross-section appearance	6.91^{a} ± 0.44	7.37^{b} ± 0.62	7.25^{b} ± 0.38
Colour	4.88^{a} ± 0.21	6.14^{b} ± 0.18	6.57^{c} ± 0.25
Smell	7.93 ± 0.62	7.56 ± 0.74	7.74 ± 0.77
Taste	8.29^{ab} ± 0.76	8.61^{b} ± 0.51	7.88^{a} ± 0.45
Texture	7.35 ± 0.89	7.76 ± 0.62	7.80 ± 0.92
Overall acceptability	7.03^{a} ± 0.20	7.87^{b} ± 0.32	7.56^{b} ± 0.35

Means within rows bearing different letters are significantly different at P<0.05

CONCLUSIONS

Sex of respondents had no significant influence on the consumers attitudes towards meat produced in different production systems, Age and education level influenced different attitudes of consumers. Group B had the highest scores for smell and taste. As a result of conducted sensory analysis, sausages from variant B (commercial broilers and nacked neck) had the highest scores for smell and taste and were selected as the best of compared samples, with overall scores of 7.87.

ACKNOWLEDGMENTS

This paper is a part of the Project EVB: TR – 31033 financial supported by Ministry of Education and Science of the Republic Serbia.

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CONSUMERS' ATTITUDES TOWARDS FULL-FAT FOOD PRODUCTS AND THEIR LOW-FAT ALTERNATIVES

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ABSTRACT: Understanding consumers' buying habits and motives for selecting high-fat products and their low-fat alternatives is useful in launching new reduced-fat food items.
In order to collect information on the most commonly chosen fat-reduced products available on the Serbian market and identify motives for their consumption, a survey among 250 randomly selected consumers in Vojvodina, Serbia was conducted. The participants could have chosen between high- and low-fat milk, yoghurt, margarine and mayonnaise and different statements referring the apprehension of these alternatives. The study also assessed the socio-demographic influence on motivation for choosing full- or low-fat food versions. The data were analysed using chi-square analysis.
In contrast to mayonnaise, participants chose low-fat milk, yoghurt and margarine in preference to their full-fat version. The results also showed that taste was the infinitely preferable attribute for choice of full-fat food, while the healthiness factor was the most crucial for selection of low-fat products. Different age as well as educational level groups showed different preferences to taste, while the consumption of low-fat food due to healthiness was more frequent among women.
The obtained results indicated the necessity to improve the taste of low- or reduced fat products in order to be competitive with its high-fat alternative.
Key words: Consumers' attitudes, low-fat, full-fat products, questionnaire

INTRODUCTION

Modern human nutrition is often related to an increasing amount of reduced fat, low-fat or light products on the market (Visschers and Siegrist, 2010). According to numerous previous researches, it was found that consumers rated taste of reduced-fat products as less pleasant than products labelled as regular-fat (Stubenitsky et al., 1999) or than reduced-fat without label (Solheim, 1992). Moreover, according to Kähkönen and Tuorila (1998), reduced-fat labels decrease people's expected pleasantness of products in comparison to regular-fat labels. The choice of food represents complex process which is influenced by a number of factors related to the product (intrinsic and extrinsic), the consumer (knowledge, beliefs, attitudes) and the consumption context (occasion, cultural environment) (Jaeger and Rose, 2008; Mela, 1999; Pollard et al., 2002). Moreover, knowledge, beliefs and attitudes to foods are the results of cultural background, eating habits and constant flow of information in everyday life (Lappalainen et al., 1998). The most important aspects which influence food choice is health and weight control. Also, motives for food choice can be related to consumers' personality and lifestyle, sensory pleasure, ideological reasons, price or familiarity (Brunsø et al., 2004; Lindeman and Sirelius, 2001). Moreover, food choice is often influenced by gender, age, social class and income (Johansen et al, 2011).
The aim of this work was to identify consumers' motives for choosing reduced-fat or fullfat products and to determine their preferences between reduced fat milk, yoghurt, margarine and mayonnaise and their full-fat versions.

MATERIAL AND METHODS

Consumers' attitudes investigations

A questionnaire was developed to collect data concerning consumers' profile, their eating habits and motives for choosing full-fat or reduced-fat product. Also, the consumers were asked to choose between various fat replacements levels of milk, yoghurt, mayonnaise and margarine which they most commonly consume. The data collection comprised 250 consumers in Vojvodina region, both in urban and suburban areas. Special attention was given to include different socio-demographic categories of consumers.

Statistical analysis

Collected data were analyzed using descriptive statistics and Pearson $\chi 2$-test, at 0.05 significance level. The statistical analyses were performed using the Statistical Analysis System software package Statistica 10.0 (Statsoft Inc., SAD).

RESULTS AND DISCUSSION

Distribution of demographic characteristics of the 250 randomly selected consumers is presented in Table 1.

Table 1. Profile of tested conumers

Characteristic	Category	Percentage of respondents (%)
Gender	man	34.4
	woman	65.6
Age group	< 30	35.2
	30 - 50	37.2
	> 51	27.6
Education	Primary and high school	40.4
	University	59.2
	no answer	0.4
Household size	1 - 2	35.6
	over 2	62.4
	no answer	2
Monthly household income	< 500 €	33.2
	500 – 1000 €.	47.2
	> 1000 €	18.8
	no answer	0.8
Profession related to nutrition and/or health	yes	15.6
	no	84.4
Body mass index (kg/m^2)	< 18.5	4
	18.5 - 25	56
	> 25	40

According to consumers' profile, it can be observed that percentage of respondents in all categories, except the BMI < 18.5, were more or less evenly distributed, which gave the representative number for giving the conclusions regarding consumers attitudes, motives and preferences. However, the obtained BMI data, which was extremely low for category < 18.5 was in accordance to the investigation performed by "Institut za javno zdravlje Srbije" (Gudelj-Rakić, 2008).

According to Budimović et al. (2010) milk consumption in Serbia was found to be roughly 180 l per person and according to data obtained by Statistical Office of the Republic of Serbia the consumption of margarine was about 2.6 kg per person in 2009 (http://webrzs.stat.gov.rs/WebSite/), while consumption of mayonnaise in Balkan countries, in 2009 and 2010 was cca 500 g per person (www.nielsen.com).
Firstly, consumers were asked how frequently they chose reduced fat versions of different food products available on the market. Accordingly, it was determined that low-fat milk, yoghurt and margarine were bought more frequently than mayonnaise (Figure 1). Moreover, it can be concluded that the answer "never" was at least frequently used for low-fat margarine. This might be due to the fact that consumers perceive margarine as a product which contains significant amount of saturated fats. Namely, according to investigation of Wansink (2003), acceptability of certain products is not only related to decrease in its energetic value but also to product type and how unhealthy is that product perceived even without decreasing its fat content. However, in the case of mayonnaise, which is a product with high fat content, the majority of tested consumers (35%) decided not to consume it at all.

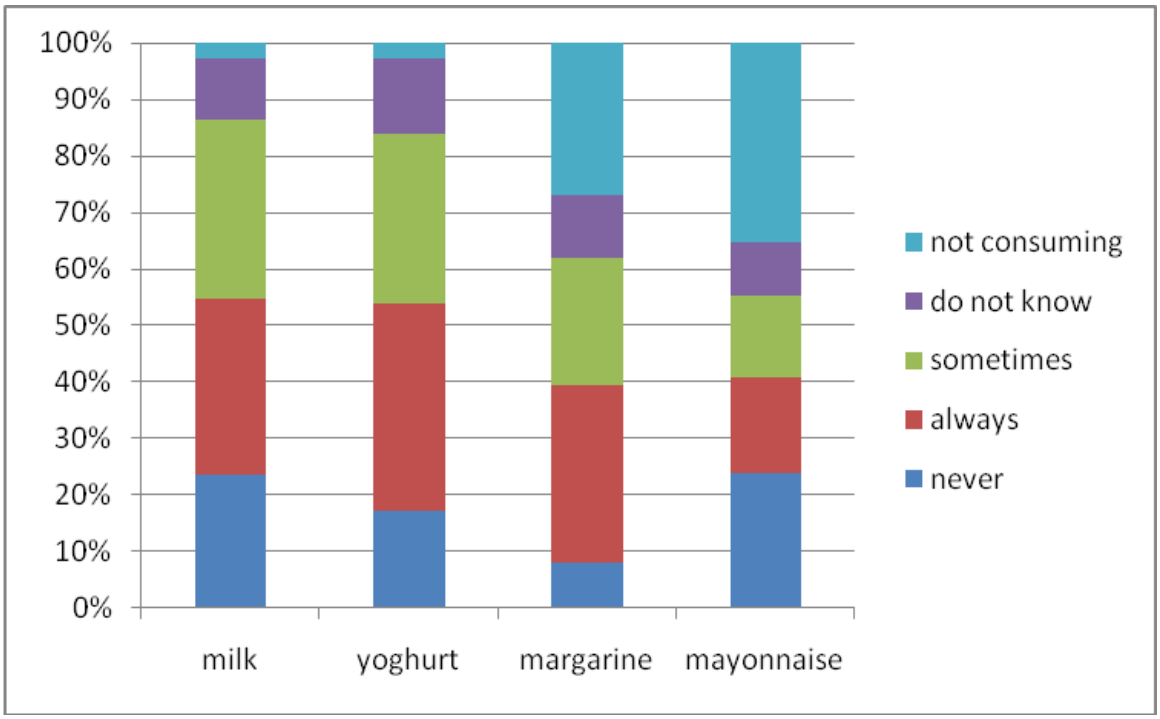

Figure 1. The presence of examined food products with reduced fat content in daily nutrition

The reasons and agreement percentages for consuming full-fat products or their low-fat alternatives are presented in Table 2.
The most important factor in consuming full-fat products was its taste followed by its availability in the supermarkets, its favourable price, etc. The most frequent answer for consuming low-fat products was that they were healthier, followed by helping in reducing weight, recommendation by a friend or from the information given by the mass media, way of life style, etc.

Table 2. Statements used in sorting test in evaluation the reasons for consuming full fat and lowfat products

Statements pro full fat products	%	Statements pro low-fat products	%
It tastes good	47.10	It tastes good	3.87
It looks nice	0.36	It looks nice	0.64
It has pleasant texture	4.35	It has pleasant texture	0.33
It is cheap	6.89	It is good value for money	2.26
It is easily available in shops and supermarkets	8.33	Recommended by medical staff or nutritionist	4.19
It is healthier	0.36	It is healthier	33.87
Not consuming	20.29	Not consuming	10.97
Do not know	12.32	Do not know	10.32
		It suits my lifestyle	8.39
		It helps me control my weight	14.52
		Recommendation by friend or information given by he mass media	10.64

According to Pearson χ2-test, at 0.05 significance level, it was determined that different age and education level groups showed different preferences to taste of full-fat products. Namely, younger people and those with the higher educational level marked the taste of the full fat product as a crucial factor in consuming these products. However, it was also determined that women consume more frequently low-fat products due to its health effects than the men do, which was in agreement with the findings of Rozin et al. (2002) and Steptoe et al. (1995).

CONCLUSIONS

The results of this study showed that between full-fat milk, yoghurt, margarine and mayonnaise and their low-fat alternatives consumers preferred all reduced fat products except the mayonnaise. Moreover, it was determined that the taste was most dominant factor in consuming full-fat products, whereas the health effect was for low-fat products. Also, different age groups as well as educational level groups expressed different preferences to taste, while the consumption of low-fat food due to healthiness was more frequent among women in comparison to men. Since it was found that taste is most important parameter in consuming full-fat products, it can be concluded that taste of low-fat products must be improved in order to be competitive with its high-fat counterparts.

ACKNOWLEDGEMENTS

This research is a part of the project funded by Ministry of education and science, Republic of Serbia (Project No 46001).

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DESCRIPTIVE SENSORY ANALYSIS AND CONSUMER ACCEPTANCE OF INDUSTRIALLY MANUFACTURED STEVIA-BASED FRUIT JUICES

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ABSTRACT: The growing consumer concern with the obesity and diabetes has led to the quest for alternative sweeteners that can replace sucrose. A sweetener that fulfills many of the consumers' demands is steviose. It is natural, intense and with zero calories. Steviose or steviol glycosides accumulate in the leaves of *Stevia rebaudiana*, a plant native to South America, and commonly known as stevia. In 2008 the Food and Drug Administration approved GRAS status for purified stevia extracts while in November 2011 the steviol glycosides were declared to be safe for use in foods and beverages in all EU countries.
In this study, steviol glycosides were used as sucrose substitute in industrially manufactured apple and peach juices. The manufacturing process of the stevia-based juices followed the regular procedure of juices containing sucrose as sweetener. Pasteurized juices were aseptically packaged in 250-mL containers and stored. As an essential component of a product development process a quantitative descriptive sensory evaluation was conducted to assess the color, sweetness, texture, aroma, aftertaste and other attributes relevant for these types of fruit juices. The overall acceptance of the juices was tested by consumer panelists according to the 5-point hedonic scale.
Key words: *stevia, steviol glycosides, sugar substitute, apple juice, peach juice*

INTRODUCTION

The leaves of *Stevia rebaudiana* Bertoni, also known as stevia, contain a group of compounds identified as steviol glycosides. More than 100 compounds have been identified in *Stevia rebaudiana*. The most commonly known compounds are the steviol glycosides, particularly stevioside and rebaudioside A, which are also the most abundant (Wölwer-Rieck, 2012).
Stevia glycosides possess valuable biological properties and their consumption could be beneficial for human health. They are noncariogenic and non-caloric sweeteners. Regular consumption of these compounds decreases the content of sugar, radionuclides, and cholesterol in the blood, improves cell regeneration and blood coagulation, prevent ulceration in the gastrointestinal tract, and can act anti-inflammatory and antihypertensive (Lemus-Mondaca et al., 2012). Studies conducted with patients proved steviosides effective in diminishing plaque formation (Blauth de Slavutzky, 2010).
Another application of stevioside is as scavenger of reactive oxygen species. Recent findings point to a prominent role of sugars or sugar-like compounds as an oxidative stress defense in plants (Stoyanova et. al., 2011).
Oliveira et al. (2012) reported recently on using stevioside as a substitute for sugars in Malay apples. In a first stage, high calorie sugars (sucrose, fructose and glucose) are partially removed from the fruit samples and in a second stage, low calorie sugars (stevioside and rebaudioside) are incorporated to the fruit to maintain its sweetness. However, such substitution does not seem economically feasible.
In 2008 the Food and Drug Administration approved GRAS status for purified stevia extracts. Although stevioside and extracts of *Stevia rebaudiana* leaves were commercially available and used as sweetener for foods and beverages in Japan, USA, and several South American countries, until recently it was not approved in the EU (Hermann, 2009). In November 2011,

the final hurdle in the EU regulatory process was lifted and steviol glycosides were declared to be safe for use in foods and beverages in all EU countries. Steviol glycoside sweeteners have been assigned the number E-960 for labeling purposes (Scardigli, 2011).
Until recently, not much data were available on the practical application of stevioside in foods and beverages. Furthermore, there is a lack of detailed information on the stability of the stevioside during different processing and storage conditions as well as its interaction with other food ingredients (Kroyer, 2010).
For the judgment of the fruit juices, a sensory evaluation is crucial. Whether the juices will be accepted or not depends on the integration of the consumer's perception of their characteristics into overall impression of quality. Although very important, chemical, physical and microbiological tests cannot provide this type of information. The objective of this study was to use steviol glycoside as sucrose substitute in industrially produced apple and peach juices and to evaluate its effect on the taste and overall acceptability of the juices by the consumers.

MATERIAL AND METHODS

Apple and peach juices

Apple and peach juices were manufactured from the fruit pulp as commercial products following the recipes of the company (Devolli group). Pasteurized juices were aseptically packaged in 250-mL containers and stored. Originally, both types of juices contain sucrose as sweetener. A commercial product Rebaudioside A with 97% purity, a product of NOW-foods, USA, was used in the experiments with steviose as substitute for sucrose. The production of all juices, standard and with steviose, was on industrial scale.

Sensory evaluation of juices

The methods used throughout the sensory evaluation of the juices were discrimination test, quantitative descriptive analysis and hedonic test (Lawless and Heymann, 1998). Sensory attributes were scored on a scale from 1 to 5 points, where higher score meant more intense attribute. A total of 92 untrained panelists, 42 women and 50 men participated in the study, all from middle class background and age range between 21 and 68. Before the evaluation started, they were briefed on the use of the sensory evaluation techniques. Each panelist tasted every sample. One sample for both descriptive analysis and hedonic test was used. The juice samples were presented in plastic cups of 150 mL. About 50 mL of juice were poured into coded cups just prior to sensory evaluation and served at room temperature. The samples were assigned three digit random codes for identification.

Statistical analysis

Descriptive statistics and one-way analysis of variance (ANOVA) was performed on the parameters to evaluate significant differences between the means at 95 % confidence interval ($p \leq 0.05$) according to Tukey's test using Minitab 15 statistical software.

RESULTS AND DISCUSSION

The sensory assessment of the juices started with a discrimination, paired comparison test. The panelists got two samples; one sample was regular juice, and the other one contained stevioside instead of sucrose. The "chemical" difference between the juices was sensory perceivable to all panelists who noticed the difference both in apple and peach juices.
The sensory properties of the apple juices are presented in Figure 1. The apple juice with sucrose tasted sweeter and fresher than the apple juice with stevioside. The apple juice with stevioside was evaluated as overpasteurized, and surprisingly with higher color score and less pronounced aftertaste than regular apple juice. The aroma attribute was almost the same for both samples.

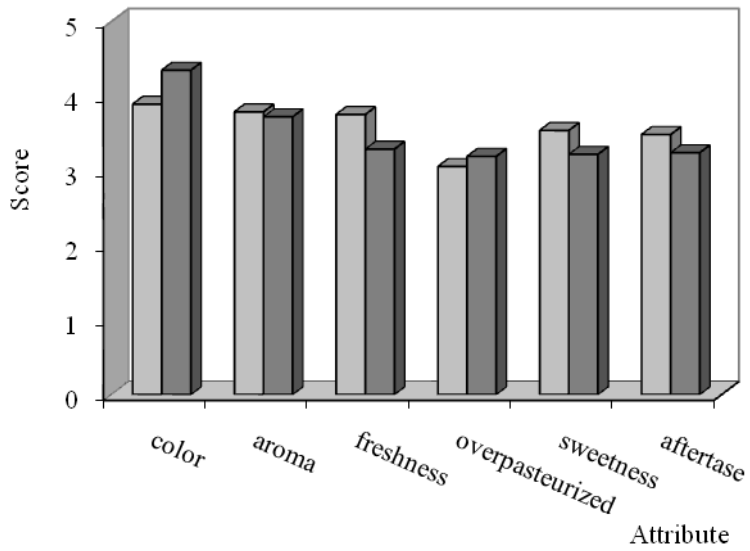

Figure 1. Descriptive sensory analysis of standard apple juice (□) and juice with stevia (■)

As can be seen from Figure 2, color and over-pasteurization of peach juice with sucrose and peach juice with steviol glycoside do not differ significantly. The peach juice sweetened with sucrose was evaluated with higher scores for aroma, freshness, sweetness, but also for aftertaste.

To better illustrate the differences of selected sensory attributes of the juices with stevia, apple juice with stevia and peach juice with stevia, the average attribute intensities of both samples are depicted in the spider web graph (Figure 3). It can be clearly seen that the apple juice with stevia has been perceived as significantly sweeter, fresher and with intenser aroma than the peach juice with stevia. However, the apple juice with stevia had more pronounced aftertaste than the peach juice with stevia. Only the feeling of over-pasteurization was the same for both juices.

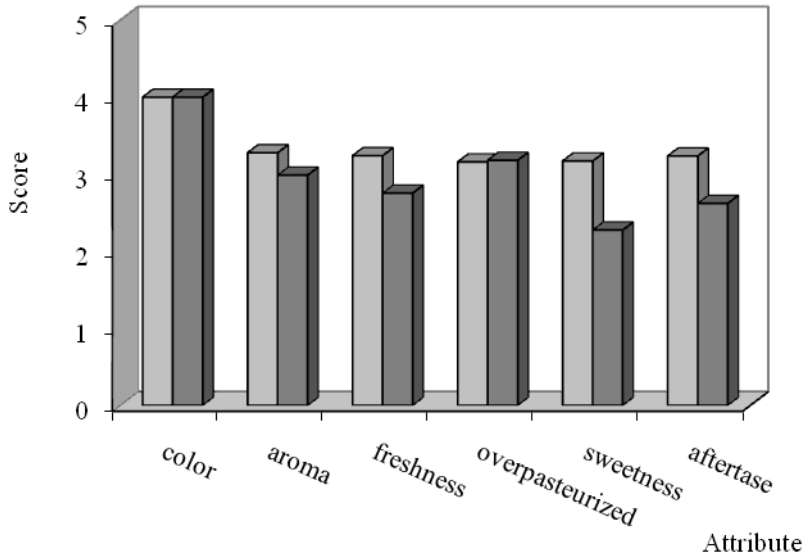

Figure 2. Descriptive sensory analysis of standard peach juice (□) and juice with stevia (■)

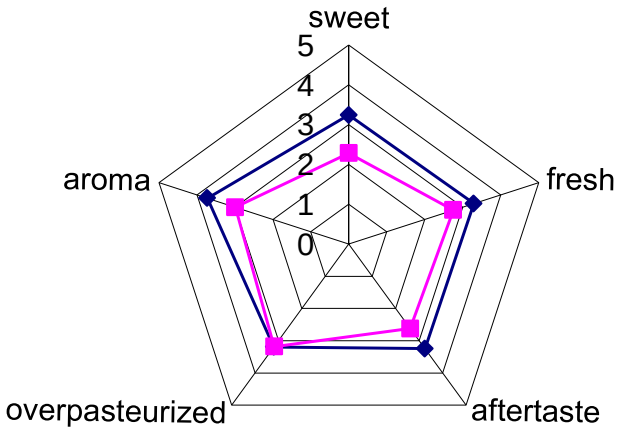

Figure 3. Spider web graph of apple (◆) and peach (■) juices with stevia

The overall acceptance of the juices was tested according to the 5-point hedonic scale used to assess liking or disliking of the panel group (Table 1). 32% of the panelists labeled the apple juice with sucrose as “like very much” followed by 25% of the panelists who gave this attribute to the apple juice with stevia. With 37% of the panelists who labeled peach juice with stevia with “neither like nor dislike”, and 17% of them who chose “dislike”, the peach juice with stevia displayed the lowest level of acceptance. The percentage of panelists who labeled the juices with “dislike” varied from 8% (peach juice with sucrose) to 4% (apple juice with sucrose).

Table 1. Hedonic judgment of the juices manufactured with and without stevia

Hedonic judgment		**Apple juice**	**Apple juice with stevia**	**Peach juice**	**Peach juice with stevia**
Nr.	Attribute	Panelists (%)			
1	Dislike	4	7	8	17
2	Neither like nor dislike	7	21	15	37
3	Like slightly	20	18	28	19
4	Like moderately	36	29	27	17
5	Like very much	32	25	22	10

CONCLUSIONS

The interest in natural sweeteners has significantly increased over the last decade.
The stevia-based juices manufactured at industrial scale were evaluated using descriptive sensory analysis. The overall acceptance of the juices was tested by consumer panelists according to the 5-point hedonic scale. Apple juice containing stevioside was accepted by the panelists while they were reluctant towards peach juice with stevioside.
The future work should be also focused on educating the consumers on the health benefits of this sweetener.

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ENHANCEMENT OF WINE SENSORY QUALITY WITH YEAST STRAINS

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ABSTRACT: Wine varietal aroma is one of the most important parameters of sensory quality and it is influenced by vintage, temperature and volume of alcoholic fermentation and several other vitivinicultural practices. In recent years the importance of yeast starter culture used for alcoholic fermentation has been highlighted. The aim of the present study was to investigate the influence of three commercial wine yeast starter cultures on aroma and chemical composition of Sauvignon Blanc wine together with three different fermentation volumes and two fermentation temperatures. The main chemical parameters, volatile thiols (GC-MS), metoxypyrazines (HS-SPME GC-MS), glutathione (HPLC-FLD) and hydroxycinnamic acids (HPLC-DAD) were determined in produced young wines. Sensorial evaluation of young wines was also performed. The results showed significant differences in the concentrations of some measured parameters and in the sensorial quality of the wines with regard to the yeast strain, volume and temperature of the alcoholic fermentations. It can be concluded that all three parameters could be an important tool for producing wines with enhanced varietal aroma and better overall quality.
Key words: *oenology, wine, Sauvignon Blanc, yeast starter culture, alcoholic fermentation, sensory quality*

INTRODUCTION

Sauvignon Blanc is a wine variety with numerous precursors for aromatic compounds, which define its typical varietal aroma. One of important aromatic compounds in Sauvignon Blanc wine are volatile thiols with tropical fruity aroma, reminiscent of passion fruit, grape fruit, mango and such. The most important volatile thiols in wine are 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA). They possess a very strong aroma with perception thresholds low as 3 ng/l for 4MMP, 60 ng/l for 3MH and 4 ng/l for 3MHA. Usual contents of volatile thiols in Sauvignon Blanc wines are 4-40 ng/l of 4MMP, 200-5000 ng/l of 3MH and 0-500 ng/l of 3MHA (Tominaga et al., 1998). Grape must contains non-volatile, non-aromatic precursors for volatile thiols 4MMP and 3MHA, which are released to become volatile during alcoholic fermentation with yeast enzyme lyase (Darriet et al., 1995). Volatile thiol 3MHA is formed from 3MH through the action of the yeast ester forming enzyme, alcohol acetyl transferase. Different yeast strains have a different ability to release volatile thiols 4MMP and 3MH from grape must and to convert 3MH into 3MHA, which is of great importance for wine sensory quality (Swiegers et al., 2006, 2009). Studies have shown that higher fermentation temperatures can increase content of volatile thiols in wine (Masneuf-Pomaréde et al., 2006).
Another important varietal aromatic compound of Sauvignon Blanc wine is the group of methoxypyrazines, which are responsible for the green character of wines produced in cooler climates. The most important methoxypyrazines in must and wine, deriving from grapes, are 3-isobutyl-2-methoxypyrazine (IBMP) and 3-isopropyl-2-methoxypyrazine (IPMP). Their content in must and wine mostly depends of vitification factors and vintage. In concentration around their perception threshold (2 ng/l for IBMP) they have a positive contribution to varietal aroma and complexity of wine (Lacey et al., 1991).
Glutathione (GSH) (γ-L-glutamyl-L-cysteinylglycine) is one of the most important antioxidants in wine, since it protects it from oxidation and by that prolongs its shelf life and preserves its aromatic potential (Rollini and Manzoni, 2006). Content of GSH in wine is largely dependent

of its content in grapes and the way of pressing the grapes, but in recent years numerous studies confirmed the importance of yeast strain used for fermentation on content of GSH (Du Toit, 2007). At the beginning of fermentation the content of GSH decreases, because yeasts are using it as a nitrogen source. Towards the end of fermentation and in the first few months of wine maturing on lees, the GSH is released back into the wine with yeast autolysis (Dubourdieu and Lavigne-Cruege, 2004). Selection of appropriate yeast strain for fermentation therefore greatly influences the final content of GSH in wine, as different strains have different ability to preserve, synthesize and release GSH (Du Toit, 2007; Lavigne et al., 2007).
The aim of the presented study was to investigate the influence of yeast strains, volume and fermentation temperature on chemical, aromatic and antioxidative composition of Sauvignon Blanc wine and on sensory quality as well.

MATERIAL AND METHODS

Sauvignon Blanc grape must was obtained from a winery in Podravje winegrowing region of Slovenia, where grapes were destemmed, crushed and pressed in inert atmosphere. Grape juice was settled over the night at 6 °C, without any further treatments. Must was divided into 25, 35 and 2500 l tanks as shown in Figure 1. Two fermentation temperatures were used – optimal (adjusted) temperature of each yeast strain and the temperature of the cellar itself. The two experiments (first in 35 l tanks and second in 2500 l and 25 l tanks) were held in two cellars. Wine yeast strains (starters), used in this study, are listed in Table 1.

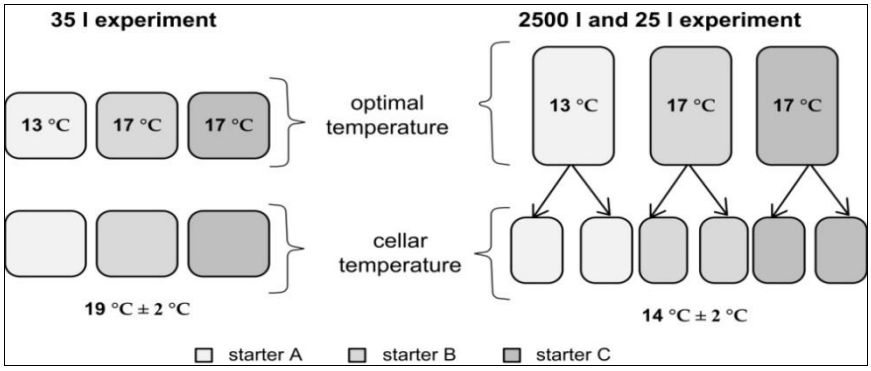

Figure 1. The schematic presentation of the experimental design

Table 1. Yeast strains used in this study

Starter culture	Commercial name of the yeast strain	Yeast strain	Producer	Dosage (g/l)
A	VIN 7 Lalvin QA23®	*S. cerevisiae* *S. bayanus*	Anchor Yeast, Cape Town, South Africa Lallemand S.A., France	0.15 + 0.15
B	Level2™ TD	*T. delbrueckii* (1st level) *S. cerevisiae* (2nd level)	Lallemand S.A., France	0.25 + 0.25
C	Exotics SPH	*S. cerevisiae*/ *S. paradoxus*	Anchor Yeast, Cape Town, South Africa	0.30

After alcoholic fermentation no fining was performed on young wines, the samples were taken from the tanks and stored in a freezer at -20 °C. In those samples the concentrations of volatile thiols, metoxypyrazines, and hydroxycinnamic acids were determined within 6 months. The determination of the main chemical parameters and glutathione was performed immediately after alcoholic fermentation. After sampling wines were racked with addition of SO_2 in concentration of 50 mg/l.

Determination of main chemical parameters
The main chemical parameters in wine (reducing sugars, alcohol, total acidity, volatile acidity and pH value) are determined by the recommended methods of European Union (Commission regulation (EEC) 2676/90 and Commission regulation (EC) 355/2005).

Determination of glutathione content
The determination of glutathione content in wine was done by the method described in Janeš et al. (2010). An Agilent 1200 Series HPLC with fluorescence detector and high-performance auto sampler for on-line derivatization controlled by Agilent Chemstation Rev. B.03.01 from Agilent Technologies (Palo Alto, CA, USA) was used for glutathione detection and quantification. Separation was performed at 25 °C using a Synergi Fusion-RP 80A column (4 µm, 150 mm × 2.0 mm i.d.) from Phenomenex (Torrance, CA, USA).

Determination of hydroxycinnamic acids
The determination of hydroxycinnamic acids content in wine was done by the method described in Vanzo et al. (2007). An Agilent 1100 HPLC with DAD connected to an Agilent NDS ChemStation (Agilent Technologies, Palo Alto, USA) was used for hydroxycinnamic acids detection and quantification. Separation was performed using an ODS Hypersil C18 column 2.1x250 mm (5 µm) with an ODS Hypersil guard column 2.1 × 20 mm (5 µm) (Agilent Technologies, Palo Alto, USA).

Determination of methoxypyrazines
The determination of methoxypyrazines IBMP and IPMP in wine was performed using modified and optimized method by Parr et al. (2007) described in Lisjak et al. (2011). A headspace solid phase microextraction (HS-SPME) with gas chromatography and mass spectrometry (GC-MS) was used for methoxypyrazines detection and quantification. Extraction was performed using divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber. Separation was performed using series of columns HP-1MS and HP-INNOWAX (Agilent Technologies, Palo Alto, CA, USA) and the detection was done using mass spectrometer with selective ion monitoring mode.

Determination of volatile thiols
The determination of volatile thiols 4MMP, 3MH and 3MHA was in wine was performed using modified and optimized method by Tominaga et al. (1998) described in Lisjak et al. (2011). A GC-MS was used for volatile thiols detection and quantification. Separation was performed using HP-INNOWAX column (Agilent Technologies, Palo Alto, CA, USA) and the detection was done using mass spectrometer with selective ion monitoring mode.

Sensory evaluation of young wines
Sensory evaluation of young wines was made two months after end of alcoholic fermentation with a group of five experts who are well experienced in wine evaluation. Wines were coded and served at 12 ºC. An evaluation test (0-5 points) was used for the following attributes: tropical fruity flavour, green pepper flavour, fermentation flavour and overall quality.

RESULTS AND DISCUSSION

Basic chemical parameters of young wines as well as content of glutathione, hydroxycinnamic acids and methoxypyrazines are presented in Table 2. It can be seen that the reducing sugars content did not differ much between wines, with the exception of wines fermented with starter C, which had the highest content in almost all cases. There were no differences between wines in alcohol content and pH value. Wines fermented with starter A had the highest contents of total and volatile acidity in all cases, while the lowest content of volatile acidity was determined in wines fermented with starter B. Fermentation with starter A at higher (cellar) fermentation temperature and smaller volume resulted with an increase in wine volatile acidity.

Table 2. Main chemical parameters and glutathione (GSH), hydroxycinnamic acids (HCA) and methoxypyrazines (IBMP and IPMP) content in young wines

Volume	**35 l**						**2500 l**			**25 l**		
	Yeast strain/combination											
	A	**A***	**B**	**B***	**C**	**C***	**A**	**B**	**C**	**A***	**B***	**C***
Reducing sugars (g/l)	1.58	1.65	1.35	1.15	2.20	2.05	1.25	1.80	1.75	1.50	1.50	11.75
Alcohol (vol. %)	10.92	10.75	10.92	10.85	10.85	10.78	9.49	10.25	10.51	10.53	10.34	10.03
pH (/)	3.36	3.38	3.37	3.37	3.38	3.40	3.31	3.31	3.37	3.31	3.32	3.33
Total acidity (g/l)	8.50	8.63	7.61	7.62	7.63	7.29	7.59	7.42	7.21	7.68	7.06	7.09
Volatile acidity (g/l)	0.99	1.25	0.48	0.47	0.55	0.55	0.68	0.33	0.59	0.90	0.40	0.59
GSH (mg/l)	13.1	10.6	15.8	15.3	11.1	10.7	16.4	20.8	14.3	13.3	17.2	9.7
HCA (mg/l)	84.33	89.46	87.88	86.97	66.22	76.10	69.06	73.19	63.40	72.94	76.05	76.39
IBMP (ng/l)							2.35	2.46	2.43	2.90	3.15	3.07
IPMP (ng/l)							nd	nd	nd	nd	nd	nd

**denotes cellar fermentation temperature. Results for 25 L volume are given as mean (n=2); nd – not detected*

The content of GSH was the highest in wines fermented with starter B in all cases, which might be a contribution of a non-*Saccharomyces* yeast strain in the starter culture B. The lowest contents of GSH were determined in wines fermented with starter C. Higher GSH content was determined in wines fermented at optimal temperature and in a larger volume. On the contrary higher hydroxycinnamic acids (HCA) content was determined in wines fermented at higher fermentation temperature and smaller volume. Also in most cases wines fermented with starter C again had the lowest content of HCA.
The concentration of methoxypyrazine IPMP was below the limit of quantification (1.6 ng/l) in all investigated wines. Higher contents of methoxypyrazine IBMP were determined in wines fermented in smaller volume at higher temperature. Yeast starter cultures did not affect on the content of methoxypyrazines.

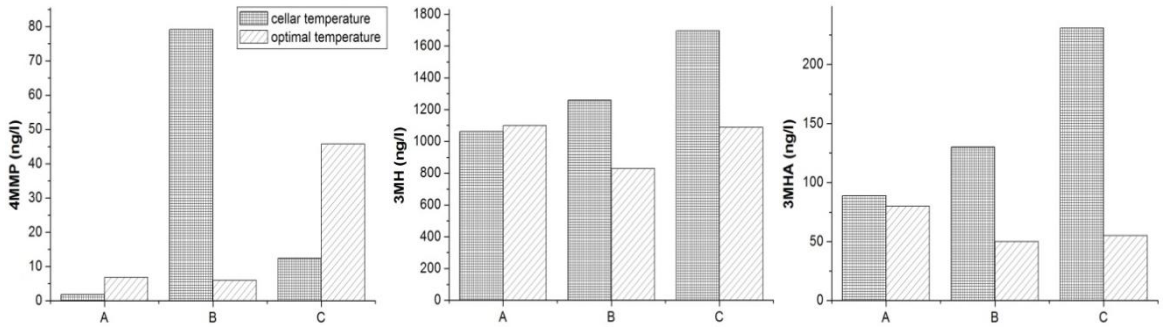

Figure 2. Content of volatile thiols (4MMP, 3MH and 3MHA) in young wines, produced by different yeast starter cultures (A, B, C) and different fermentation temperature in 35 l tanks

Fermentation temperature greatly influenced the content of volatile thiols in produced young wines (Figure 2). Lower temperatures increased the content of 4MMP in wines fermented with starter A and C, while wines fermented with starter B had an extremely high

concentration of 4MMP at higher temperature. Higher temperature also resulted in increase of 3MH and 3MHA in almost all cases, especially in wines fermented with starter C with the highest concentration of mentioned thiols. Fermentation temperature itself had the least influence on volatile thiols in wines fermented with starter A. At optimal temperature wines fermented with starter A had the highest contents of 3MH and 3MHA, while wines fermented with starter C had the highest contents of 4MMP. The conversion rate of 3MH into 3MHA was higher at higher fermentation temperature, especially in the case of starter C. At optimal temperature starter A had the highest conversion rate of 3MH into 3MHA.

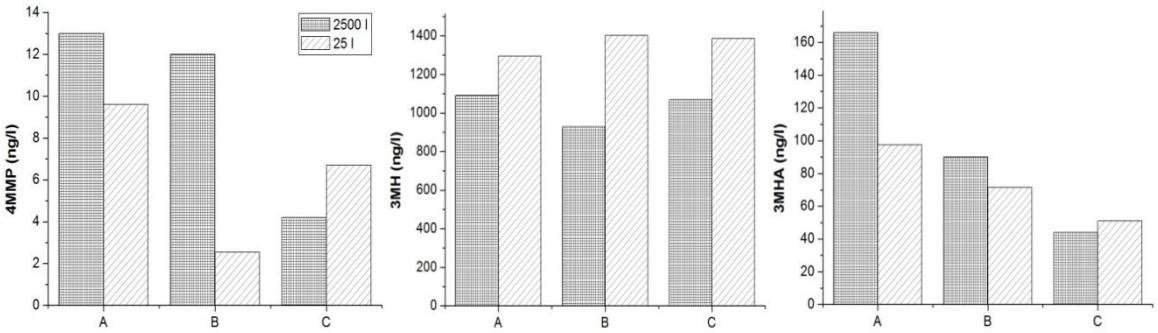

Figure 3. Content of volatile thiols (4MMP, 3MH and 3MHA) in young wines, produced by different yeast starter cultures (A, B, C), in different fermentation volume and at different fermentation temperature (2500 l tanks - optimal temperature, 25 l tanks - cellar temperature).

Influence of volume of alcoholic fermentation on content of volatile thiols is shown in Figure 3. Larger fermentation volumes resulted in higher contents of 4MMP and 3HA in wines fermented with starters A and B, while the fermentation with starter C in larger volume had the opposite effect. On the other hand, smaller fermentation volumes resulted in higher content of 3MH in all cases. In larger fermentation volumes wines fermented with starter A had the highest contents of all three volatile thiols, while the lowest contents of 4MMP and 3MHA were detected in wines fermented with starter C. In smaller fermentation volumes again wines fermented with starter A had the highest contents of 4MMP and 3MHA, but in this case the smallest content of 3MH.

Sensory analysis performed on wines fermented in 35 l tanks showed that higher fermentation temperature resulted in wines with lower ratings of all evaluated sensory attributes. Between wines, fermented at optimal temperature, the once fermented with starter A were rated the highest for attributes tropical fruity flavour and fermentation flavour. The highest rated for attribute overall quality were wines fermented with starter B. In the case of wines, fermented in 2500 l, wines fermented with starter A were again rated highest for attributes tropical fruity flavour, fermentation flavour and overall quality, while attribute green pepper flavour was rated highest in wines fermented with starter B. On the other hand, wines fermented in 25 l were relatively similarly ranked for evaluated sensory attributes. With a small difference, wines fermented with starter C were ranked highest in all evaluated sensory attributes.

CONCLUSIONS

As different fermentation parameters did not influence much on basic chemical parameters, with the exception of higher volatile acidity in wines fermented with starter A, they had a greater impact on varietal aroma and antioxidative state of wine in terms of GSH and HCA content. The content of volatile thiols and GSH in wines varied greatly in dependence of the used yeast strain, which also reflected on sensory quality of wine that was in general the best in wines fermented with starter A. Higher fermentation temperature resulted in higher content of volatile thiols, but lower content of GSH, which led to lower sensory quality of wines due to oxidation. Larger volume of fermentation generally resulted in higher content of volatile thiols (4MMP, 3MHA) and GSH, but lower content of methoxypyrazine IBMP. It can be concluded

that yeast starter culture, volume and fermentation temperature have an influence on wine varietal and fermentation aroma and therefore on overall wine quality.

ACKNOWLEDGEMENTS

The authors thank Dr. H. Baša Česnik, Dr. K. Lisjak, Dr. A. Vanzo, L. Janeš and Z. Zupančič for individual analyses and B. Kobal (Ptuj Wine Cellar) for the must. The present work is a part of an applied research project “The impact of technological processes in maintaining the aromatic potential in technology of wine production” (No. L4-2042) financed by SlovenianResearch Agency.

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FOOD PRODUCT DEVELOPMENT AS OPPORTUNITY FOR SUCCESS OR SURVIVAL IN THE MARKET

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ABSTRACT: The aim of this paper was to give an overview of up-to-date papers covering sensory evaluation and consumer preference research that can form a basis for user-oriented food product development in Small and Medium Enterprises (SMEs). The paper points out the most important factors with impact on food products fail, that may arise as a consequence of not matching consumers' needs. Collecting of appropriate information concerning consumers' needs and expectations are essential requirements of a consumer-oriented product development. Consumer's sensory tests can provide sensory information related to food product quality and consumers preference, useful and important for product quality control and improvement. The elements with impact on consumers' food products preferences are identified and discussed. Success and survival of SMEs on the market could be expected if producers identify and satisfy current and future needs of consumers. Management and policy makers of the enterprise should support development and implementing sensory programs within an enterprise, and use it as a tool for ensuring quality of product and competitive food manufacturing. Consumer-oriented food product development should be considered as a tool for building competitive advantage and long-term enterprise success in the market and for prevention of negative changes in product quality and acceptability, consumers' complaints and product rejection.
Keywords: *food product development, sensory evaluation, consumers*

INTRODUCTION

Small and Medium Enterprises (SMEs) are an important contributor to growth and employment in the European Union, and they are much greater in number than large companies. Enterprises face the challenge of being competitive on a global scale and SMEs continue to face with limited capacity to expand to markets beyond their home country (Commission Recommendation 2003/361/EC; European Commission Proposal, 2011/0394 COD). SMEs are often said to be responsible for driving innovation and competition in many economic sectors. As major factors that distinguish the SMEs as growers and non-growers Hansen and Hamilton (2011) emphasize: their opportunistic perceptions of the environment; controlled growth ambition within their own needs and financial, intellectual or personal capabilities; a developed culture of innovation referring to new ways of doing things commonly associated with new products or processes; flexibility that refers to agile approaches to products, product markets and customer services; and use of extensive private business networks. SME performance is directly associated with and affected by the procurement system, by logistics, and particularly by the general attitude and behaviour adopted by enterprises towards their customers and suppliers (Orfanos et al., 2010). Considering the effect of various management practices on performance of small business enterprises in manufacturing industry Gadenne (1998) found that small retail and manufacturing firms may be able to successfully compete in the open marketplace with good quality, pricing products lower than competitors, acquiring knowledge of competitors' activities, and with better customer relations.
Most SMEs play a key role as innovators in EU economy, although they are not exercising research and development activities, as officially defined. Innovation in SMEs relies to a large extent on qualified entrepreneurs and employees and on cooperation with suppliers

and customers (UEAPME, 2009). The economic success of the food processing enterprises in a competitive environment depends on their capability to improve their products, identify and satisfy consumers' requirements (Grunert et al., 2008; van Trijp and van Kleef, 2008). Transformative consumer research is studying the role that consumption plays in the major social problems of our day, with the goal to do practical research that can be used by consumers' activists, policy makers and businesses to improve consumer well-being (Ozanne et al., 2011). Nowadays consumers' requirements regarding food products are more diverse than ever before, thus challenging the producers to design and produce food products that are perfectly tuned to the wishes of individual consumers (Benner et al., 2003). For SMEs is very important to assess its strategic position, challenges and to identify opportunities performing continuous improvement of user-oriented product quality, food product development, increasing productivity and competitiveness and staying in a mature market.

The aim of this paper was to give an overview of up-to-date papers covering sensory evaluation and consumer preference research that can form a basis for user-oriented food product development in SMEs. The paper points out the most important factors with impact on food products fail, that may arise as a consequence of not matching consumers' needs. The paper is organized as follows. First, an overview of up-to-date papers covering sensory evaluation and consumer preference research has been given. Second part of the paper offers a brief review of the literature related to the food product development, with impact on activities which SME should assess for improving current methods and practices in a consumer-oriented food production and products fail prevention. Third part of the paper emphasizes importance of the food product quality control and quality control sensory programs implementing, as a tool for ensuring high and consistent quality of final products and competitive food manufacturing in SME. Finally, some conclusions are provided.

Sensory evaluation and consumers' research

Collecting of appropriate information concerning consumers' needs and expectations are essential requirements of a consumer-oriented product development. Researches on consumer behaviour, demand, expectations and consumption of food generally are important the same as analysis and understanding of consumer preferences (Van Kleef *et al.*, 2006; Gielens and Steenkamp, 2007; Miljkovic and Effertz, 2010). The key determinant of success in product development is the degree to which a product coincides with consumer needs and expectations (Sijtsema *et al*., 2004; Bahamonde et al., 2007). Consumer's sensory tests can provide sensory information related to food product quality and consumers preference, useful and important for product development and quality control.

The areas or growth and opportunities for improvement companies' success in the market include awareness of the importance and support for quality control sensory programs within an organization and involvement of sensory scientists in it (Munoz, 2002; Moskowitz et al., 2006). Adequate information exchange throughout the complete food production chain is a vital element in the realisation of an efficient and effective product development process in SMEs. The sources of information and knowledge, mostly used for innovative ideas by SME in the food area, are in their direct surroundings (Benner et al., 2003).

Food producers must know that consumers have concerns about influence of the foods they eat on their health. Their personal values, especially those related to sensory quality characteristics and nature of food may be strong predictors of consumers' concerns about food preference, acceptability and choice (Linnemann *et al.*, 2006; Worsley and Lea, 2008). A range of factors have influence on special categories of consumers or children, youth and teen population lifestyles, food choice and diets (Pescud and Pettigrew, 2010; Roberts and Pettigrew, 2010; Luchs et al., 2011; Mason et al., 2011). The visual design parameters should be considered in the food product development process. The packaging colour and shape may influence food product quality evaluations. Package appearance is important, it may be specifically designed with particular symbolic meanings, related to the selected sensory properties and impact on overall product evaluations and price expectations (Becker et al., 2011).

Consumer-oriented products have to deliver nutrition performance, function, convenience, but most importantly, sensory characteristics related to the product acceptability (Moskowitz and Hartmann, 2008). A quality control function is an operation closely aligned with the food manufacturing process, with emphasis mainly on instrumental and chemical analyses. Most companies have well defined and established quality control programs, but no quality control sensory programs. The quality of food products is correlated with their sensory properties, and implementing quality control sensory program may have a vital role in the consumer-oriented food production (Munoz, 2002). The experienced specialists in sensory analysis and quality control, with strong personal networks can play a fundamental role in supporting SMEs, creating stable and continuing relations communicating between SMEs and universities, institutions and science laboratories.

Food product development

Small and Medium Enterprises (SMEs) were important developers of radical innovations (Massa and Testa, 2008). Enterprise should assess its current situation and implement new or improve current methods and practices. Food product development is a continuous process that is of great concern to producers, because of the fact that tomorrow's consumers needs are changing with time and they may not want the products that were in great demand yesterday (Costa and Jongen, 2006; Linnemann *et al.*, 2006; Grujić and Cantalejo, 2009).

Small enterprise may achieve long-term survival through timely strategic and management changes, encouraging close relationships among core shareholders and core customers (Ng and Keasey, 2010). In food product development, SME should established food quality and sensory standards and incorporate it in specifications for final products QC. It should include results of consumers' preference research. Food product development process in SMEs can use available external sources of inputs and expertise from suppliers, research institutes, universities, partner companies, and customers.

Innovation and product developments in SMEs are permanent process driven by small entrepreneurs and their staff. These kinds of activities include new and improved products, new processes, new markets and management concepts and play an important role for economic growth. New food products often fail, because they are not designed according to consumers' wishes or not produced efficiently. SMEs need qualified staff or adequate support services to design and handle products development projects, especially if bigger changes in production process are involved. That could be solved using external sources of expertise from research institutes, universities and customers. The reasons for the low innovation rate in the food industry are numerous. The qualitative customer requirements should be translated into design parameters of the product during development process. Parameters are measurable attributes and quality determinants of the food product. The approach of Mattsson and Helmersson (2007) illustrated a method to cluster consumer attribute preferences and to transform that text about certain food product, spontaneously written by consumers, in directions for work on food product development. In order to reach a stage of sustainable development, firms need crucial resources, such as a large enough market, an accepted product or service, purchasing customers and a supportive network. Furthermore, the firm has to be able to utilize these resources in a satisfactory way that is, through its motivation, competence and organization (Norrman and Bager-Sjögren, 2010). Each of mentioned factors have role in matching consumers' needs and impact on food products success or fail.

Quality of food product as competitive advantage

Many SMEs are facing with lack of capacity, human and financial sources for food product development, but consumers require and expect quality and safety of food products in the market (Costa and Jongen, 2006; Mattsson and Helmersson, 2007; Grujić and Cantalejo, 2009; Pescud and Pettigrew, 2010). Most companies have well defined and established quality assurance and quality control programs to pursue and maintain the products' quality and prevent negative changes in product quality, consumers' complaints or product rejection. The emphasis is mainly on instrumental and chemical analyses, many enterprises have

some type of quality control sensory programs, but only a few have sound quality control sensory programs (Munoz, 2002). Management and policy makers of the enterprise should support development and implementing sensory programs within an enterprise, and use it as a tool for ensuring high and consistent quality of final products and competitive food manufacturing.
The research work of Arulselvan et al. (2008) provides a practical approach for classify food industry products, to understand characteristics of the food industries and structure of the food industry market. Success and survival of SMEs could be expected if producers identify and satisfy current and future needs of consumers. Status consumption is the consumption of goods for displaying status. It could be used as quality characteristic for products development, s obzirom na to da as modern status orientation has a positive impact on status consumption and satisfaction with life in general (Nguyen and Siok, 2011).
The success of a product is determined by the profit a company and by selling the product (Benner et al., 2003). For the products survival on the market, the consumers have to buy the product once, and if it fulfils certain expectations they will buy it again. The product also has to be better in fulfilling these requirements than similar products available on the market.

CONCLUSIONS

Success and survival of SMEs on the market could be expected if producers identify and satisfy current and future needs of consumers. Collecting of appropriate information concerning consumers' needs and expectations are essential requirements of a consumer-oriented product development. Consumer's sensory tests can provide sensory information related to food product quality and consumers preference, useful and important for product development and quality control. Management and policy makers of the enterprise should support development and implementing sensory programs within an enterprise, and use it as a tool for ensuring quality of product and competitive food manufacturing. Consumer-oriented food product development should be considered as a tool for building competitive advantage and long-term enterprise success in the market and for prevention of negative changes in product quality and acceptability, consumers' complaints and product rejection.

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SENSORY EVALUATION AS TOOL IN QUALITY IMPROVEMENT OF BOILED CHICKEN SAUSAGE

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ABSTRACT: Food product quality and stability during storage is very important for the successful manufacturing. It is known that different factors during processing have impact on quality characteristics of final product. The aim of the study was to investigate influence of selected food additives with antioxidant and stabilising functional characteristics on quality of finely comminuted boiled chicken sausages "parizer" type, during storage. Samples were produced in industrial conditions, five experimental model samples with selected additive blends added separately to each: (a) 0.04% GUARDIAN Rosemary Extract 08 (Natural rosemary extract, E471, E472a, E1520); (b) 0.10% (based on the fat content) GRINDOX 539 Antioxidant (E304, E306, E322, rapeseed oil); (c) 0.30% GRINDSTED Carrageenan CC 310 (E407, E410); (d) 0.30% GRINDSTED MEATLINE 345 A Emulsifier and Stabiliser System (E401, E516, E470a, E450); (e) 0.30% GRINDSTED MEATLINE 333 Stabiliser System (Carob germ flour, E466) and (f) control sample, according to the producer's specification. The samples had adequate chemical composition. Sensory evaluation of selected sensory characteristics was done 7 days and 35 days after samples production: (1) by the descriptive sensory analysis - scoring method, and (2) by discriminatory sensory analysis - ranking method, after assessment and comparison of the overall quality of sausages model samples. The results of the sensory analysis indicated differences between model samples dependent on added additives. On the basis of descriptive sensory evaluation results, it was found that the best quality of compared had sausage sample produced with addition 0.30% GRINDSTED MEATLINE 345 A Emulsifier and Stabiliser System (E401, E516, E470a, E450). Discriminatory sensory analysis – ranking confirmed that finding.
Key words*: sensory analysis, chicken sausages, quality*

INTRODUCTION

Food industry has an important position in the processing industry, especially when we talk on the manufacturing of meat products. Meat and meat products are very important ingredients in human nutrition because they are an excellent source of protein with a well-balanced composition of amino acids, B vitamins and mineral components (Borowski, 2007).The most important tasks of meat industry include the development of products that identify and satisfy consumer needs and expectations and have relatively long shelf life.
Quality control is an essential component of any food processing business. Accurate and objective chemical and physical methods should be used in the food quality control, but despite of progress in this area, the human senses are still more efficient than instrumental methods in evaluation of flavour and texture, and provide more rapid responses. The reliability of information obtained through the human senses can be increased by selection and training of assessors, providing suitable environment for evaluation, and using appropriate testing procedures. Therefore, sensory evaluation should be an integral part of product quality defining and controlling process (Krysztofiak, 2005; Savanović and Grujić, 2008; Vasilev et al, 2011). The importance of sensory analysis is great and it is applicable in different areas: for improve quality of the products throughout the development process, for describe products sensory characteristics and for comparing products with competitors' products (Leppard et al., 2004; Živković et al, 2011; Maughan et al., 2012). Methods of sensory evaluation are unavoidable and applied for development of meat products and as tool for detailed sensory characteristics describing. Assessors have to be tested and trained

according to the standard methods (ISO 8586-1:1993). In such cases, during realisation of sausages quality improvement process, different aspects of sensory evaluations are considered, using visual, olfactory, gustatory and palpatory techniques that enable detailed judgment of particular quality characteristics (Leppard et al., 2004; Giboreau et al., 2007; Savanović and Grujić, 2008). That approach could create a base for designing of model for products quality improvement in the meat industry.

Today the market offers a large number of meat products from the category of boiled sausages. All generations of consumers like to eat and appreciate quality of finely comminuted boiled chicken sausages "parizer" type as they have pleasant and mild flavour. It is known that different factors during processing have impact on quality characteristics of final product. Product quality could be defined in many ways. For sensory quality of parizer, important attributes are: outside appearance, cross section appearance, colour of cross section, smell, aroma and taste, and consistency (Savanović and Grujić, 2008). Sensory characteristics of sausages depend on the type of meat, ways and length of storage of meat, then the aroma and chemical composition of meats, as well as used additives and spices (Bilska, 2007; Grujić et al., 2008). In aim to improve the quality of meat products various food additives can be used (Serdaroglu and Yildiz-Turp, 2004; Bilska, 2007; Hong et al., 2008; Garcıa-Garcıa and Totosaus, 2008).

The aim of the study was to investigate influence of selected food additives with antioxidant and stabilising functional characteristics on quality of finely comminuted boiled chicken sausages "parizer" type, during storage.

MATERIAL AND METHODS

Boiled chicken sausages "parizer" type, were used as a model-product in the experiment. Samples were produced in industrial conditions according to the producer's specification. The following ingredients were used (percentage shown in descending order): mechanically deboned chicken meat (60%), lean chicken meat (15%), vegetable oil, water, salt, isolated soy protein, dextrose, spices, stabilizers, antioxidants, flavour enhancer, preservative. Five experimental samples were produced with selected additives added separately to each (Table 1). The sixth model-sample (P6) is produced according to the manufacturer's recipe and it was also used as a control sample. Sausages were packed in tight artificial casings (diameter 60 mm; average mass of sausage pieces was 500g) and after heat treatment were chilled and labelled appropriately. Until the moment of test samples were stored in a dark and cold place, at +4°C to +8°C.

Table 1. Selected additives blends used for producing of the boiled chicken sausages

Selected additives blends (and ingredients)	**Sausage sample code and additives blends content (% w/w)**					
	P1	**P2**	**P3**	**P4**	**P5**	**P6**
GUARDIAN Rosemary Extract 08 (Natural rosemary extract, E471, E472a, E1520)	0.04	-	-	-	-	-
GRINDOX 539 Antioxidant (E304, E306, E322, rapeseed oil)	-	0.10*	-	-	-	-
GRINDSTED Carrageenan CC 310 (E407, E410)	-	-	0.30	-	-	-
GRINDSTED MEATLINE 345 A Emulsifier and Stabiliser System (E401, E516, E470a, E450)	-	-	-	0.30		-
GRINDSTED MEATLINE 333 Stabiliser System (Carob germ flour, E466)	-	-	-	-	0.30	-

** - quantity expressed based on the amount of fat in the finished product*

Analysis of chemical composition was carried out in Laboratory for food analysis on Faculty of Technology, University of Banja Luka. The following parameters were determined according to AOAC (2006) procedures: moisture content (drying at 105 °C to constant mass),

the fat content (according to the Soxhlet method), protein content (according to the Kjeldahl method), the content of sodium- chloride (according to the Mohr method), and the content of nitrite (expressed as $NaNO_2$, according to the method ISO: 2918:1975), the content of phosphates (expressed as P_2O_5, according to the spectrophotometric method ISO: 13730:1996).

Individual sensory evaluation was carried out by 10 trained assessors (ISO 8586-1:1993), in the Laboratory for sensory analysis of food on Faculty of Technology, University of Banja Luka, 7 and 35 days after production. Six labelled different model samples (five samples in which selected additives were added and the sixth control sample) were twice delivered to assessors during the sensory analysis. Water and cubes of white bread were served to assessors for cleaning the mouth between samples evaluation. Descriptive sensory analysis - scoring method, and discriminatory sensory analysis - ranking method were used for evaluation of sausages quality.

In initial preparation of sausages sensory evaluation by the scoring method (ISO 4121:2003; Radovanović and Popov-Raljić, 2001), coefficient of significance (Cs) was determined for each selected sensory attribute (sum of them are 20). Appropriate Cs was multiplied with score given after sensory evaluation of each selected attribute (in scale from 5 for very good quality, to 1 for very bad quality). Addition of all results of evaluated sensory attributes multiplied with Cs, gives overall score, expressed as percentage of maximum possible products quality, or 100% for the best quality. For boiled sausages the most important sensory attributes were evaluated: odour, aroma and taste (Cs=6), colour of cross section and consistency (Cs=4), outside appearance and cross section appearance (Cs=3). The scoring forms with description of the sensory attributes and possible defects for each quality level were delivered to the assessors (Grujić et al., 2008; Savanović and Grujić, 2008).

Discriminatory sensory analysis – ranking of six model samples of sausages were realised on the basis of to the 20 judgements of overall acceptability: the most acceptable (on the first place), the least acceptable (on the last place). The rank sums were determined and statistical comparisons (α=0.05) were carried out by the Friedman test (ISO 8587:2006) for demonstrating and recognition by assessors differences among evaluated samples.

RESULTS AND DISCUSSION

The average chemical composition of the tested sausage samples is shown in the Table 2. The content of the tested compounds in all samples were in accordance with the requirements defined by the Regulation on the quality of poultry meat products (Official Journal SFRJ No. 55/91), valid in Bosnia and Herzegovina, where production was realized.

Table 2. The average chemical composition of the boiled chicken sausages

	Sausages sample code					
	P1	**P2**	**P3**	**P4**	**P5**	**P6**
Moisture (%)	58.61	58.50	58.06	57.65	58.71	58.07
Fat (%)	20.12	21.03	18.49	17.87	17.36	19.23
Proteins (%)	16.09	15.51	15.89	15.40	15.66	15.71
NaCl (%)	2.64	2.45	2.49	2.31	2.43	2.57
Phosphate (P_2O_5) (%)	0.29	0.27	0.29	0.28	0.29	0.27
Nitrite (mg/100g)	0.025	0.024	0.021	0.025	0.026	0.027

The results of the sensory analysis indicated differences between model samples depending on added additives. Outside appearance of all six chicken sausage samples were on acceptable quality level, without any noticeable defects (Table 3). Selected food additives with antioxidant functional characteristics had positive impact on colour of finely comminuted boiled chicken sausages “parizer” type during storage. After 7 days of storage it was found that between produced samples the most acceptable colour score (4.70) had sample P1, produced with addition of natural rosemary extract, and after 35 days of storage the most acceptable colour score (4.85) had sample P2, produced with addition of ascorbyl palmitate

and tocopherols (Table 3). These results are in agreement with previously published papers, confirming the positive effect of antioxidants on colour stability of meat products (Serdaroglu and Yildiz-Turp, 2004; Bilska, 2007; Mancini et al., 2007).
The scores for evaluation of the cross section appearance of sausage samples were relatively lower than expected. Partial annular layers of colour shade and larger pieces of ingredients were observed at the cross section of some sausage samples, and affected sensory scores, as shown in Table 3. That did not significantly affect on the overall acceptability of the product, but it is more pleasant to eat particles of sausages ingredients of uniform size. For cross section appearance, after 7 days of storage, scores were from 4.55 for P3 sample, to 3.85 for P5 sample. Similar average values of these quality characteristic were assigned after 35 days of storage (Table 3).

Table 3. Results of the descriptive sensory evaluation for the boiled chicken sausages

		Average scores for quality parameters (n=20 assessments)					
Sample code	**Storage time (days)**	**Outside appearance (Cs=3)**	**Cross section appearance (Cs=3)**	**Colour of cross section (Cs=4)**	**Odour, aroma and taste (Cs=6)**	**Consistency (Cs=4)**	**Evaluated overall quality* (%)**
P1	7	5.00	4.25	4.70	4.15	4.30	83.95
P2	7	5.00	3.95	4.45	4.05	3.85	84.75
P3	7	5.00	4.55	4.55	4.25	4.35	89.75
P4	7	5.00	4.30	4.50	4.45	4.70	91.40
P5	7	5.00	3.85	4.60	4.10	4.45	87.35
P6	7	5.00	4.40	4.30	4.05	4.10	86.10
P1	35	5.00	4.35	4.75	4.30	3.95	80.05
P2	35	5.00	3.90	4.85	4.30	4.00	87.90
P3	35	5.00	4.40	4.25	4.20	4.45	88.20
P4	35	5.00	4.25	4.70	4.40	4.85	92.50
P5	35	5.00	3.95	4.25	4.15	4.40	86.35
P6	35	5.00	4.65	4.45	4.20	3.90	87.55

*Cs – coefficient of significance; * Evaluated quality – percent of maximum possible quality*

Among the many sensory characteristics, the odour and taste sensations take particular place when eating food products. Manufacturing of model chicken sausages samples and a control sample were performed according to the same recipe, from identical basic ingredients, and that results in approximately uniform odour, aroma and taste of all samples (Table 3). Most samples were evaluated by score higher than 4 and with the comment that the aroma of the product were weaker than expected, and odour of fat and other ingredients were slightly more perceptible than it is desirable.
The consistency of processed meat products depends on the structure of the matrix formed by the proteins gel, the solutes and particles entrapped in the gel and the moisture content. Therefore, the matrix forming depends on factors such as protein water binding ability, salt content, pH, fat content and the addition of nonmeat ingredients (Flores et al., 2007). Comparing the consistency of the control sample, and produced models of chicken meat sausages, differences in the structure of the products after 7 days of storage were determined by sensory evaluation. Consistency of control sample was defined as mild finely granular, powdered, with gentle glimpses and hardly noticeable release of fat droplets. Compact mass and chewiness of the control sample were satisfactory. Consistency of samples P1, P2 and P3 were evaluated as appropriately and described as a persistent, slightly elastic-plastic and contents did not release water or fat under pressure (Table 3). The best scores for consistency were assigned to the samples P4 and P5 (Table 3). Consistency of these samples were described as elastic, sausages were sturdy and juicy, connectivity of components were good and compact, chewiness were satisfying, neither too hard nor too soft. Scores of consistency, after 35 days of storage, showed that the control sample P6 produced according to the producer's specification, had the lowest value of the mean score, followed by the sample P1, while samples P3, P4 and P5 produced with additives with of

emulsifying and stabilizing functional properties, were evaluated with the highest scores (Table 3). Hydrocolloids are currently added to meat products to improve cooking yields, increase moisture retention and modify consistency of product (Garcıa-Garcıa and Totosaus, 2008). Many studies have been carried out on these products and their application in comminuted food products. They are added as another gelling system to improve yield, textural properties and also reduce cost of the meat formulation. The addition of potato starch has a positive impact on the consistency, improvement the emulsion stability and reduction the jelly and fat separation in cooked meat products (Flores et al., 2007; Aktas and Genccelep, 2006). Hong et al. (2008) have found that carrageenan and glucono delta lactone (GDL) have high binding characteristics and minimal discoloration of restructured pork, even if a low salt concentration was used. Garcıa-Garcıa and Totosaus (2008) reported that carrageenan-locust bean gum interaction improved texture and water retention, with only a minor effect on sausage colour.

Comparing the overall quality of the produced sausages, expressed as a percentage of the maximum quality, after 7 days of storage was found that the sample P4 had the highest overall quality (91.40%), followed by the sample P3 (89.75%), and the samples P5, P6, P2 and P1 (Table 3). The similar quality of products was evaluated after 35 days of storage. Sausage P4 was the best with 92.50% of the expected overall quality, then the samples P3, P2, P6, P5 and sample P1 with relatively the lowest score (80.05%), as shown in Table 3. It should be noted that all samples had relatively high scores for overall quality and can be characterized as products with very good quality level.

Table 4. Rank sums calculated based on the overall acceptability of boiled chicken sausages

Sample code	Rank sums after 7 days storage	Rank sums after 35 days storage
P1	70	58
P2	85	79
P3	70	80
P4	58	47
P5	73	85
P6	64	71

Ranking is method for discriminatory sensory evaluation with the aim of placing a series of test samples in rank order. Based on the overall acceptability of the sausages model samples ranking were realised after 7 and 35 days of storage, for recognition by assessors of differences among the samples (Table 4). Critical table value for the Friedman test is 10.92, for the 6 samples and 20 judgments (significance level α=0.05). Calculation of the rank sums evaluated after 7 days of samples storage, results in value 5.91 for Friedman test (F_{test}<F) and indicated that there are no statistically significant differences (α=0.05) between compared products. Comparing the rank sums obtained after 7 days of storage showed the lowest value of the rank sums (58) for the sample P4 and that it had the highest quality of compared sausages (Table 4). After 35 days of storage calculated value for Friedman test was 15.43 (F_{test}>F), and it was concluded that there were statistically significant difference (α=0.05) in the quality between compared samples of sausages. After 35 days of storage sample P4 had the rank sums 47 and the best quality comparing to the other samples (Table 4). Sample P4 with modified composition by adding of 0.30% additives blend GRINDSTED MEATLINE 345 A Emulsifier and Stabiliser System (E401, E516, E470a, E450), could be characterised as sausage with the best improved sensory acceptability of the compared.

CONCLUSIONS

Sensory quality characteristics of the finely comminuted boiled chicken sausages “parizer” type depends of method of manufacturing, quality, quantity, chemical composition and aroma of used meat, spices, other ingredients and functional property of food additives. The results of the sensory evaluation of sausages model samples indicated that addition of selected

additives blends could impact on quality and improve overall acceptability of sausages. On the basis of descriptive sensory evaluation results, it was concluded that the best overall quality of compared, had boiled chicken sausage sample produced with addition 0.30% additives blend GRINDSTED MEATLINE 345 A Emulsifier and Stabiliser System (E401, E516, E470a, E450). Discriminatory sensory analysis – ranking based on overall acceptability of the sausages, confirmed that finding.

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FRUIT AND VEGETABLE AS FUNCTIONAL FOOD – CROATIAN CONSUMERS´ OPINIONS

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ABSTRACT: Public perceptions relating to fruit and vegetable as functional foods was investigated using a survey. The cross-relation of their perceptions was examined according to the gender, age and educational level. The main aim was to identify the public acceptance of the concept of functional food as well as possibilities of the marketing in this filed.
In this paper a convenience sample was used and data were collected using a self-administrated questionnaire. The questionnaire was designed to assess opinions and attitudes about consumption and about fruit and vegetables as functional foods. Individuals of both genders (N = 628) aged from 19 to 65 with different education levels were interviewed. Stratification of the units from the sample has been made according to gender, age and educational structure as well as social-economic status of respondents in the sample.
The study showed that over 45.7% of female consumers are familiar with the term functional food which is more than male consumers (36.5%). Results also indicate differences regarding education level and age because the majority of younger consumers with higher education are interested to pay more (34.6%) for vegetables and fruits. The consumers consider as most important the origin (67.5%) of fruits and vegetables and their quality (77.9%). The place of sale and the brand of the product seem not to be as important as expected (49.6%). The opinions are especially divided ($p<0.05$) according age and education level and not to the gender ($p>0.05$). The results have shown that general familiarity with functional food varies systematically as a function of age, gender and education level.
Key words: *vegetable, functional food, consumers*

INTRODUCTION

Food provides essential nutrients for normal function of body, but unbalanced diet over a long time increases the risk of developing nutritional diseases and involves expensive medical treatments. Phrase “functional food” was introduced in Japan (1980s), for food products that were fortified with special constituents with advantageous physiological effects (Wootton-Beard & Ryan, 2011). Numerous study have reaffirmed that functional foods may improve the general conditions of the body (Chen, 2011; Bhat & Bhat, 2011), decrease the risk of some diseases, and could even be used for curing some illnesses (Ngo et al., 2011). It was recognized that there is a demand for these products in all age and gender groups. Functional food products typically include health claims on their label describing their benefits: for example for vegetables: "Vegetable is a significant source of fibre” (JHCI, 2002) which has been proven to provide health benefits over and above basic nutrition (Elleuch et al., 2011). Consequently, the fruit market faces great opportunities. In recent decades, research has confirmed that the consumption of fresh fruit products has a positive effect on a person’s health (WHO, 2003). The World Health Organization (WHO) recommends eating at least 400 g of fruits and vegetables per day (WHO, 2003). National recommendations for fruit and vegetable have adopted similar intake levels throughout the European Union. Examples of these governmental recommendations are five a day campaigns in the UK, Spain and France and two plus two campaign in the Netherlands. However, despite governmental intervention strategies, general fruit consumption in EU countries does not meet the

recommended level. Average consumption of vegetables and fruits in Croatia is 350 grams per day (GfK, 2011), what is under the recommendation.
The inclusion of physiologically active natural components with beneficial effects on health strengthens the nutritional value of fresh vegetables (Sanzana et al., 2011; Wootton-Beard and Ryan, 2011). A health diet should include at least five portions of fruit and vegetables a day according the Food Standards Agency (FSA, 2009) but the majority of the population do not consume the recommended portions for fruit and vegetables (FSA, 2010). Diverse fresh food like vegetable and fruits, convenient, value-added, ''healthy'' food are trends in new lifestyles. Higher incomes and consumer awareness are creating consumer demand for a year-round supply of high quality products (Onwezen and Bartels, 2011).
Responsibility for inadequate daily intake of fruit and vegetables obviously lies with the consumer, but there is also an inherent challenge to the food industry to develop new, exciting and convenient food products to help inspire people to make a positive change to their diet (Wootton-Beard and Ryan, 2011). Fresh fruit and vegetables consumption can lower the risk of diseases such as coronary heart disease and specific types of cancer and are preferable in the human nutrition regarding their richness of antioxidant what is a benefit for the public health. Antioxidants differ in their efficacy against differing substrates; some are potent free radical scavengers whilst others have stronger metal chelation effects (Niki & Noguchi, 2000). It is however widely accepted that many compounds traditionally thought of as antioxidants have important non-antioxidant functions including maintenance of redox balance (Formanet al., 2002; Hensley et al., 2000; Valko et al., 2007). It has been suggested that some molecules which are traditionally thought of as antioxidants (such as polyphenols) may also have pro-oxidant effects in certain issues (Azam et al., 2004).
Main idea of this study was to investigate familiarity with the term functional food and is the vegetable and fruit recognised as functional food.

MATERIAL AND METHODS

This research was conducted in the spring 2010 and the sample frame consisted of participants aged over 18 years (N=628; 45.7% women, 54.3% men) from Croatia (Table 1).

Table 1. Socio-demographic characteristics of the data set

Characteristic		%
Gender		
	Female	45.7
	Male	54.3
Age (y)		
	18-30	50.6
	31-50	39.6
	51-60	9.8
Education level		
	Elementary school	7.3
	High school	56.9
	University degree	37.8
Income		
	< 4.000 kn	16.6
	4.001 – 6.000 kn	29.7
	6.01 – 10.000 kn	33.1
	> 10.000 kn	20.6

1 € = 7.6 kn

Data were collected using a self-administered questionnaire distributed among respondents. The questionnaire included questions about the functional food knowledge, attitudes regarding functional food as well as the socio-demographic variables (Table 1).
From the collected data two different matrices were developed – one for those who know the meaning of functional food and the other one for those that are not familiar with the term

"functional food". From the first matrix (those who are familiar with functional food) were developed two new matrices regarding the (i) vegetable consumption and (ii) fruit consumption.
Data were analysed using SPSS (Statistical Package for Social Science, v.15). The ANOVA (Two-Factor without Replication) was used to determine differences between observed variables. Crosstabs from the descriptive statistics were used to observe interactions for at least 2 variables.

RESULTS AND DISCUSSION

In the total data set, familiarity with the term "functional food" was expressed by 40.7% (N=628) of the participants and almost 52% of them were female consumers. The female consumers were fewer in the total population of participants what is in accordance with previous publications (Markovina et al., 2011; Chen, 2011). Over 42% of interviewed Croatian consumers are familiar with the concept of functional food, what was also observed in other European countries where the familiarity with the term "functional food" is ranged from 25% in United Kingdom, Germany and France to 49 % in Belgium (Annunziata and Vecchio, 2011).
One of the aims was to determine similarities and/or differences in the gender affiliation. According ANOVA, the difference in global perception of functional food is confirmed ($p<0.001$) and is matching with results of the study of Markovina and co-workers (2011). In the paper were also investigated the differences in perception of vegetables and fruits as functional food ($p<0.05$) as well as the influence of the education and income level on the perception which food is concerned as functional food ($p<0.005$).

Table 2. Participants' opinion regarding fruits and vegetables as functional food products

	%		
Characteristic	**Fruits**	**Vegetables**	**Total (fruits & vegetables)**
Gender			
Female	28.3	17.4	45.7
Male	32.0	22.3	54.3
Age (y)			
18-30	34,8	21.2	56,0
31-50	15,1	20.6	35,7
51-60	8.3	4.0	8.3
Education level			
Elementary school	18.7	18.0	36.7
High school	36.1	20.1	56.2
University degree	3.6	3.5	7.1
Income			
< 4.000 kn	10.6	5.9	16.5
4.001 – 6.000 kn	17.3	12.6	29.9
6.001 – 10.000 kn	19,8	13,6	33.4
> 10.000 kn	11.8	8.4	20.2

1 € = 7.6 kn

The results of this study, regarding fruits and vegetables considered as functional food, are in accordance with some studies (Santosa et al., 2010) where was concluded that vegetables and fruits are staple food for most countries in the Mediterranean region (i.e. Spain, Italy, and Greece). Numerous studies have pointed out the health benefits associated with rich intake of vegetables and fruits that helps to lower cardiovascular risk (Martinez-Gonzales et al., 2004) because of their richness in dietary fibres. 39.7% from all participants consider vegetable as food with functional properties what can be related with richness in antioxidants (Forman et al., 2002; Hensley, et al., 2000) or dietary fibres (Elleuch et al, 2011). It is questionable if this attitude is a consequence related to the food choice preferences or to the nutrition richness of food.

Vegetables and fruits are considered as healthy food because this is the food which can deliver natural antioxidants and other bioactive phytochemicals in a small volume but offering a convenient method of consumption and contribute significantly to overall vegetable consumption (Wootton-Beard and Ryan, 2011). Also, high share of fruits and vegetables in the daily food intake may play an early protective role against the development of cardiovascular diseases, (CVD) as a consequence of high amounts of fibers and antioxidants (Thomson et al., 2007).

As expected, higher level of education and higher incomes are critical in classification of fruits and vegetables in the category of functional food. Most informed age group regarding fruits and vegetables as functional food is group from 18-30 years. They are young people on the peak of their working life and it is important for them to take care about their health. Those results are in accordance with those presented by Annunziata and Vecchio (2011) where the functional food users are more often more educated.

The functionality of fruit and vegetable is generally recognized by the consumers. In order to be more familiar with their buying habits the analysis of their place of purchase was tested (Fig. 1) on all age and gender groups as well as in different education and income.

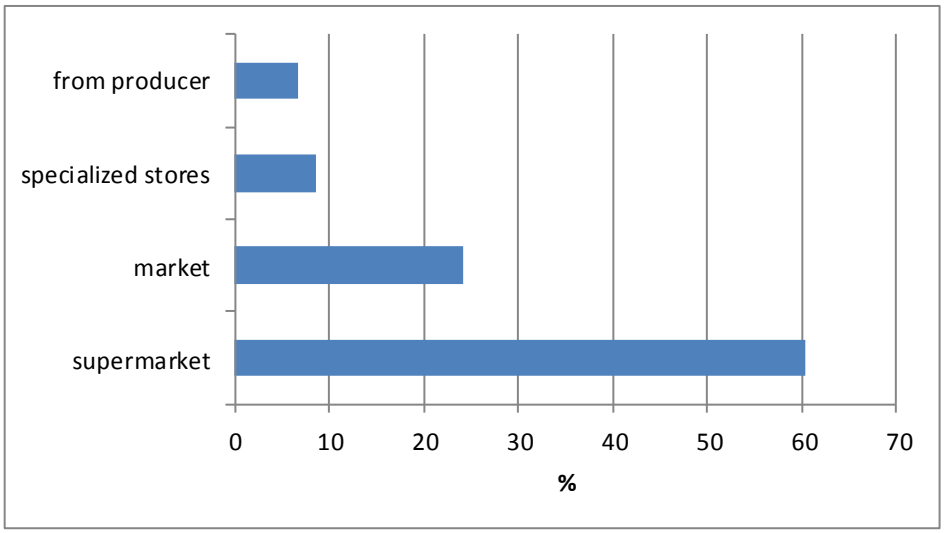

Figure 1. Consumers' preferred places of purchase of vegetables and fruits

The places of purchase of vegetables and fruits are presented in Figure 1, and, as it can be seen, supermarkets have earned the trust of the consumers (60% of the interviewed consumers believes that the vegetable and fruit purchased there has functional properties. 45.3% of buyers are not sure should they pay more for functional food products and that should be a challenge for the producers and industry.

In order to make more detailed analysis of presented result, place of purchase has been additionally analysed according to socio-economic parameters (table 3).

Female consumers are more oriented on buying on the market, specialized stores followed by supermarkets. Opposite of them man chose to by fruit and vegetables (considered as functional food products) direct from the producer (68%), what is also the first choice of those aged 31-50 (48%) and with higher incomes (6.001-10.000 kn) where almost 40% of them would prefer to by direct form the person that has produced the vegetable and fruit considering it that as functional food.

Table 3. Participants' opinion regarding place of purchase and main features of fruits and vegetables.

Characteristic	%						
	Specialized stores	Super-market	Market	From producer	Origin	Brand	Quality
Gender							
Female	55.6	49.1	56.0	32.0	77.1	51.8	65.6
Male	44.4	50.1	44.0	68.0	78.6	49.4	69.2
Age (y)							
18-30	47.4	61.2	52.7	34.0	77.5	43.9	66.5
31-50	40.5	34.9	38.9	48.0	72.9	58.1	67.9
51-60	12.3	4.0	8.4	18.0	92.0	76.0	88.0
Education level							
Elementary school	31.7	36.3	42.3	48.0	76.9	55.8	66.8
High school	66.7	56.3	54.4	47.9	78.4	47.0	68.2
University degree	1.6	7.5	3.3	4.1	79.1	51.2	66.7
Income							
< 4.000 kn	16.7	15.4	15.3	20.8	76.0	48.5	70.0
4.001 – 6.000 kn	16.7	31.5	31.3	22.9	75.4	44.4	67.2
6.001 – 10.000 kn	28.3	31.9	35.8	39.6	80.7	45.5	68.3
> 10.000 kn	38.3	21.2	17.6	16.7	77.2	66.1	63.8

1 € = 7.6 kn

Both genders have considered the origin and the quality as the most important characteristics of fruits and vegetables. This fact is especially evident by the oldest participants (92% for origin and 88% for quality).

CONCLUSIONS

Over 42% of Croatian consumers are familiar with the concept of functional food but the perception of the foods that are considered as food with functional properties differ with the socio-demographic characteristics of the consumers. Female consumers were more familiar with the functionality of food, in general.

Consumers' recognition of fruits (over 51%) and vegetables (42%) in a group of functional foods has differed, especially regarding the education level and age. Presented findings could be an indicator of a successful marketing approach in sale of vegetables and fruits. Majority of the consumers choose fruits and vegetables as a food that will improve their health, especially male consumers (54.3%) aged under 30 (56%), with finished high school (33.4%) and middle (29.9%) or higher incomes (33.4%).

The findings of this work also imply that the impact of the gender, age and education level is not negligible.

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HOW THE POPULATION'S PERCEPTIONS INFLUENCE THEIR BEHAVIOURS REGARDING THE CONSUMPTION OF FIBRE RICH FOODS

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ABSTRACT: The association between dietary fibre, health benefits and healthy food has been studied in recent years. The food industry, to accompany the strong interest shown by consumers, has placed at their disposal new products, rich in dietary fibre. In order to meet this huge interest and demand for these products, this work was elaborated, which had as main objective to evaluate the degree of knowledge of the Portuguese population about fibre and its effects on health, as well as evaluating the their consumption habits in respect of fibres. To achieve this goal an inquiry was carried out by questionnaire to 182 adults. The most relevant results indicate that only 13% of the inquired eat two meals a day with vegetables and/or salads and 9% eat at least 3 pieces of fruit. Whole grains are never consumed by 41% and 18% do so at least once a week. The vast majority (90%) of respondents have the notion that fibre intake contributes to the prevention and treatment of diseases. With this work it was concluded that respondents' knowledge about dietary fibre is insufficient, and that although they give great importance to their role in treatment and prevention of diseases, the level of intake is too low.
Key words: *fibre, food habits, fibre intake, fibre rich food*

INTRODUCTION

Food plays an important role in the maintenance of human life by providing the caloric and nutritional elements necessary for the body functions. Among these elements, dietary fiber has attracted great interest in the last decades, having been produced several studies in this area (Kendall *et al.*, 2010). The theme "dietary fiber" was one of the most attractive in nutrition and public health in the second half of the 20th century, and it was the stimulus for a large number of researches at many levels: epidemiological, physiological, analytical and technical. In that context, it was the incentive for the development of knowledge about the causes of several diseases, especially those dealing with the large intestine and diabetes, and has contributed for the governments and the food industry to establish valuable goals to a healthy diet (Cummings *et al.* 2004). Since then there have been a growing number of studies about dietary fiber, not only related to their diet aspects, but also on its economic recovery, resulting in increased knowledge of its chemical, nutritional and functional properties (Rodríguez *et al*., 2006).

At present, this issue continues to awaken interest and it is clear the growing concern that people have about health. Epidemiological studies have shown a correlation between diets rich in fibre and a lower incidence of some chronic diseases such as cardiovascular disease (Honda *et al*., 1999), colon cancer (Bobek *et al*., 2000, Honda *et al*., 1999) and breast cancer (Park *et al*., 2009). Also fiber intake has an effect on the absorption of glucose, and in general, the consumption of fibres induces a lower risk of disease (Gallaher & Schneeman, 2003).

Although it is known that dietary fibers play an important role in preventing various diseases, and that diets high in fiber, such as those rich in cereals, fruits and vegetables, have a positive effect on health (Kendall *et al*. , 2010), often this is not taken into account in the current daily intake of food. The stress of modern life, has led to increased demand for processed foods with predominance of refined products rich in saturated fats and low in

dietary fiber. The increased consumption of these foods, combined with the stress and the reduction of physical exercise, led to an increase in several human health disorders such as obesity, hypertension and heart problems (Kendall *et al*., 2010).
Besides the growing importance that has been attributed to dietary fiber, there have also emerged new sources of fiber and a better understanding of the technological functionality of fibres, which allowed offering new opportunities for its use in the food industry. In addition to the nutritional effect, the fibers can be used for economic and technological purposes as well. As technology agents, their use may range from a bulking agent to a substitute for fat (Guillon & Champ, 2000), and have been used in bakery products, beverages, candy, milk, frozen dairy products, pastas, meats and soups . In recent times, the production of products rich in fiber is "fashionable". At the technological level there has been interest in order to improve the sensory characteristics of products, so as to make them not only healthier but also more "attractive" from the organoleptic point of view.
Dietary fibres have been studied, particularly in recent years, for the positive effects that they have on people's health. To understand what is the level of knowledge that a group of the Portuguese population demonstrate about the benefits of their intake and also what are their motivations and habits of consumption, a survey was conducted and a statistical analysis of the results was consequently performed.

EXPERIMENTAL METHODOLOGY

To achieve the objectives of this study, it was conducted a survey by mix questionnaire, with open and closed questions to obtain some more qualitative information to complement the quantitative information. The questionnaire was divided into various topics in order to obtain data related to consumption habits, knowledge about dietary fiber, and ways of disseminating information, food labeling, the relationship between fiber, variety of foods and disease.
The survey was conducted between April and June 2011, having been obtained in total 182 completed questionnaires. For the treatment of data two software programs were used: SPSS (Statistical Package for Social Sciences - version 18) and Microsoft Excel (version 2007).

RESULTS AND DISCUSSION

182 people were surveyed of which 54% were female and 46% were male. Approximately 70% had between 18 and 40 years, university frequency and lived in urban areas.
To link the consumption of fiber with some of the eating habits of those surveyed, were asked questions related to weekly frequency of consumption of vegetables, salads, fruit, fast food, whole grains and number of meals outside the home. About 39% of those surveyed make more than one meal a day with a salad and/or vegetables, and only 2% do more than two. These numbers are far below those recommended by the current food wheel published by the Directorate General of Health in 2005, which indicates that we should eat vegetables at least in the two main meals every day. The results indicate that only 13% of those surveyed do satisfy this requirement. Regarding the number of times they eat meals away from home per week, 31% report eating five meals, which may be related to the number of days a week that people are normally absent from home, mostly for reasons of work or school. This is reinforced by the fact that the vast majority of those surveyed are old enough to be in working life. Approximately 90% eat meals such as "fast food" once a week or simply do not consume them al all, and this information somehow goes against the present tendency to increased consumption of such foods (Kendall *et al*., 2010). The whole foods do not seem to be part of the daily diet of respondents because 41% said not to eat whole grains and food products. One meal a day with them is made by more than 12% and 82% do not eat whole foods every day.
The results presented in Table 1 allow to verify the level of information of the regarding their knowledge about fibres.

Table 1. Relative frequency of the answers regarding knowledge of those surveyed about fiber.

	Relative Frequency (%)				
	Totally disagree	**Disagree**	**not agree or disagree**	**Agree**	**Totally agree**
1. Only plant foods have fiber	13.2	35.7	14.3	22.0	13.7
2. Foods of animal origin such as meat, eggs and dairy products have no fiber (except if added)	11.0	22.5	23.1	28.0	14.3
3. According to World Health Organization, an average adult should eat 25g of fiber per day	2.7	7.1	56.0	26.9	5.5
4. The whole foods (pasta, bread, rice, cereals, ...) have the least amount of fiber than non-integral	33.5	33.5	17.0	10.4	3.8
5. The unpeeled fruits have less fiber than peeled	34.1	39.6	15.4	7.7	1.6
6. Dietary fibers are classified into soluble and insoluble	3.3	4.4	45.1	24.7	21.4

When asked if the fibers are of animal or vegetable origin, the responses are confused, suggesting that this area is not known to the majority of those surveyed. In relation to the recommendations of the World Health Organization and the Directorate General of Health (Candeias, n.a.), the majority of respondents (56%) did not know how much fiber should be consumed each day (25 g/day). This data reveals that the importance of fiber to the diet is not known to the majority of respondents, since only 5% are completely sure of their answer. The largest quantity of fiber present in whole foods is known to 67% of respondents, revealing that there is an association between whole foods and a higher amount of fiber. The same relationship is observed with the skin of fruit because 74% of those surveyed said that the fruit with skin has more amount of fiber compared to that peeled. The answers to the last question show that 45% of people had no opinion about the solubility of fibers and equivalent percentage of respondents agree with the statement.

There are several ways to pass and give information that keep the general pubic informed about various topics, including about fibers. In this way, questions were asked about the means to get more information related to fibers and their consumption and, in the opinion of those surveyed, the means that would be most appropriate are shown in Figure 1.

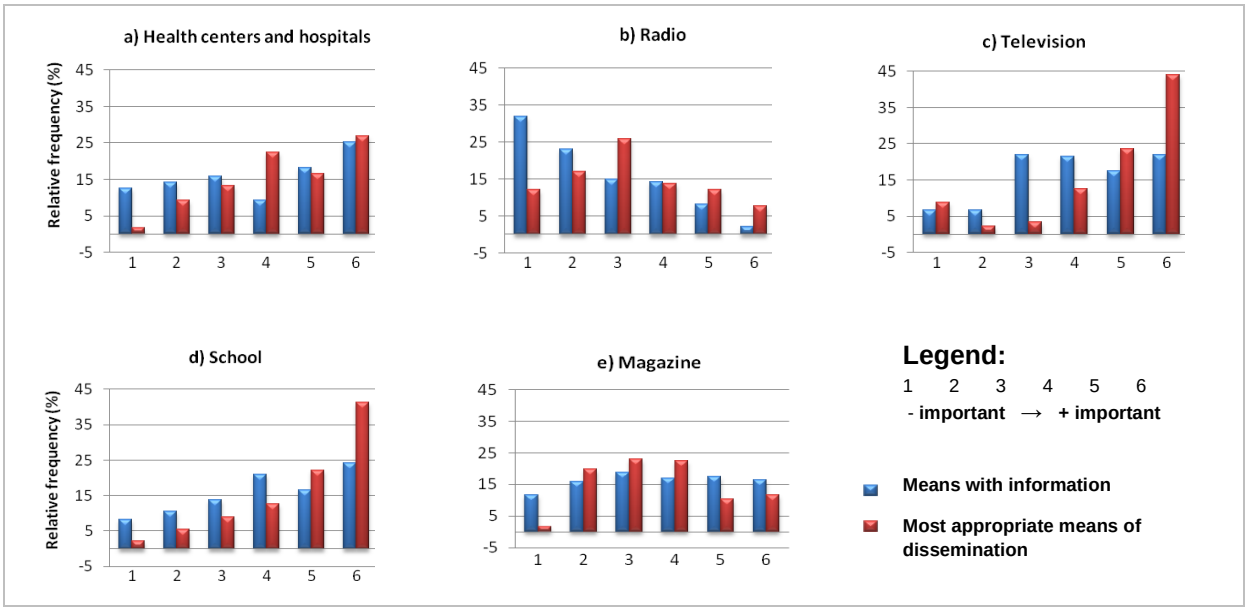

Figure 1. Means of dissemination for information about dietary fibre

From the analysis of the responses, one can highlight two media considered more appropriate to encourage the consumption of fibre: television (44%) and school (41%), but none of these was identified as where there is usually more information on fibres. In fact, the respondents considered most important the health canters and hospitals as the places with available information on this subject. To the radio was given little importance, because this media was referred less often. It should be noted that health centers, hospitals, television and school are the means of transmission of information considered the most important.

Foods have different fiber contents and some of it is not part of the natural food constitution. In this context, it was designed a group of questions in order to evaluate the respondents' knowledge about the food groups that have higher fibre contents (Table 2). The analysis of the responses indicates that respondents do not have consolidated knowledge concerning the origin of the fibers, taking into account that the fiber source is of plant origin and both the results in Table 1 and Table 2 do not evidence this in a clear way.

As it can be seen in Table 2, nearly half of the enquired responded that the fibers have nutritional value. However, this high percentage obtained may not due to effective knowledge about the recent legislation (just over one year) which indicates that the fibers have an average energy value of 8 kJ/g (2 kcal/g) (Dl nº. 54/2010 28/05), but due to the lack of information. Most respondents agreed that legumes, cereals and fruits are rich in fiber. Studies described by Slavin (2008) and Martins *et al*. (2006) among many other corroborate the opinion of those surveyed. Furthermore, the results did now allow establishing a relationship between fiber intake and the environment of living (rural or urban).

Table 2. Relative frequency of responses concerning the relation between fiber and variety of food

	Relative Frequency (%)				
	Totally disagree	**Disagree**	**not agree or disagree**	**Agree**	**Totally agree**
1) Dietary fibers have their origin only in plant foods	12.1	33.5	15.9	22.0	13.7
2) Dietary fibers have their origin only in animal foods	36.3	45.1	15.4	0.5	0
3) Dietary fiber can come from in food animals and plants	12.1	20.3	22.0	34.1	9.3
4) The fiber have calories, ie, they provide energy to the body when ingested	12.1	20.9	19.2	40.1	4.9
5) Leguminous plants (beans, peas, beans, ...), cereals and fruits are foods rich in dietary fiber	1.6	5.5	13.2	45.6	31.3
6) The consumption of dietary fiber is higher in urban than in rural áreas	18.7	37.9	31.3	7.1	1.6

Another group of questions was delineated to investigate whether the respondents relate the fibre intake with health benefits associated to some types and diseases. According to Figure 2, about 90 % of respondents agree with the fact that fibers can prevent and treat disease.

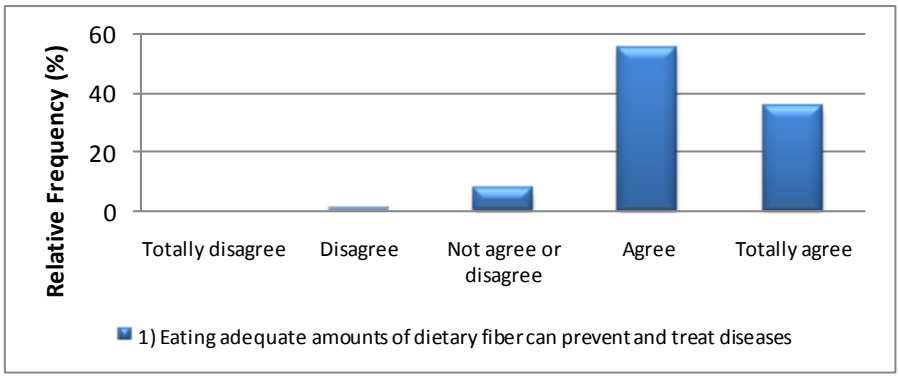

Figure 2. Relationship between fibre intake and disease

Other questions aimed that respondents could eventually establish a relationship between fiber and some diseases. The prevention and treatment of deficiency in vitamins and minerals or vision problems were the illnesses most commonly mentioned as not having to do with the intake of fibre. On the other hand, the most common diseases related to the benefits of fiber intake were: constipation; (86%), obesity (80%), cardiovascular disease and cholesterol (70%), bowel cancer (69%).

Food labels and nutrition information were also examined. These are consulted by most respondents when buying food products, however there are few that give relevance to the amount of fiber that a food product contains when making their purchase.

CONCLUSIONS

The most relevant results indicate that just over 10% of those surveyed eat two meals with vegetables and/or salad a day and at least three pieces of fruit (recommended intake to achieve the recommended intake is 25 g of fibre/day). Whole grains (bread, pasta...) also showed low levels of consumption, despite the respondents revealing knowledge about their higher fibre contents.
The consultation of food labels is of interest for the majority of respondents, about 80%, but little more than half show curiosity to know the fiber content that the food actually has.
The vast majority of respondents have the notion that fiber intake contributes to the prevention and treatment of diseases. Of the various diseases listed, the most cited were constipation (86%), obesity (80%), cardiovascular disease and cholesterol (70%) and bowel cancer (69%).
With regard to the means of dissemination for information on dietary fiber, school and television were considered the most appropriate media to encourage consumption, but in the opinion of respondents, health centers and hospitals are those where more information is at present available.
This study also revealed that knowledge about fibres and the amounts eaten should be higher than they are now. Furthermore, and despite being given high importance to the fibers in the treatment and prevention of diseases, the intake is actually still very under the recommended amounts.

ACKNOWLEDGEMENTS

The authors thank financial support to the research centre CI&DETS.

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THE EFFECT OF THE NAME OF THE TECHNOLOGY ON CONSUMER'S ACCEPTANCE – THE RESULTS OF FOCUS GROUP INTERVIEWS

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ABSTRACT: In the framework of the NovelQ EU FP6 project, the consumer acceptance of novel technologies was studied by qualitative and quantitative methods. According to the research results the name 'Pulsed Electric Field' (PEF) evokes the fear of electricity, thus not widely accepted. Prof. Dietrich Knorr, Chairman of the NovelQ Scientific Advisory Board thus proposed a new expression to replace PEF, namely „micro pulse".
The acceptance of the new terminology was studied focus group interviews. Three focus groups were organised with consumers (elderly age consumers, young adults and health conscious consumers) and one more focus group with experts of food sciences, specialised in technology, legislation, food policy, consumer sciences and nutrition sciences.
The results of the focus group interviews with consumers highlighted, that name 'micro pulse' evokes better associations than the pulsed electric field and 'micro pulse' is in no way associated with 'electricity'. Consumers expressed their definite need of getting informed about the new technologies even if this leads to distrust because of the lack of comprehensive knowledge. Consumers' judgment about the change of the technical term is diverse: according to the opinion of two focus groups, it is not misleading to use the name 'micro pulse' instead of PEF, though the electrical feature of the treatment should be mentioned. According to the respondents of the health conscious focus group, change of the name is misleading, because the aim is the reduction of consumers' fears. The most important issues rose by the expert focus group, namely that clear communication is essential for the consumers, and the electrical feature of the technology has to be expressed. The term 'pulsed electric field' is threatening and 'micro pulse' is not informative. The term 'micro electrical pulse' might be acceptable according to the experts. It is important from the market point of view a new technology that it has to offer unambiguous benefits for the consumers. A communicational campaign is needed for the introduction of novel technologies.
Key words: *food processing technology, Pulsed Electric Field (PEF), focus groups*

INTRODUCTION

Our present research was carried out in the framework of the NovelQ (Novel Processing Methods for the Production and Distribution of High-Quality and Safe Foods) EU FP6 integrated project. The aim of the Sub Project 3 was to study the consumers' acceptance of novel food processing technologies and novel products, and the perceived benefits and costs. Secondly, to develop guiding principles for the implementation of novel technologies to be used mainly SMEs in various regions in Europe.
Several findings underpinned, that comparing the Pulsed Electric Field (PEF) technology to the HPP, there are more negative attitudes and less positive attitudes towards the PEF process and products, than towards the HPP process and HPP products. (Butz et al., 2003; Cardello et al., 2007; Mireaux et al., 2007; Nielsen et al., 2009; Sonne et al., 2012).
We carried out a qualitative study on consumer attitudes towards Pulsed Electric Field (PEF) and high-pressure processing (HPP). 12 focus groups were organised with 97 adults in Slovenia, Hungary, Serbia, Slovakia, Norway and Denmark. Participants were generally positive towards these novel technologies as the processes are seen to be environmentally friendly and result in natural products with high vitamin content. Besides, consumers were sceptical toward PEF because of being uncertain about the risks. The name Pulsed Electric Field generated a fear of electricity (e.g. 'I just cannot imagine this high voltage. I'm afraid of

it. It sounds bad. Electricity is not for me.' Female, 29 years old, Hungary). In addition, PEF products were seen as negative as they are believed to trigger allergic reactions. Another difference between the two technologies is that only PEF is associated with the well known processing methods like microwave oven and irradiation, which technologies were not favourable for the consumers. (Nielsen et al., 2009)
The likelihood of choice for novel processed (PEF, HPP) apple juice with conjoint analysis by was also examined by our network 609 consumers in Norway, Denmark, Hungary and Slovakia. The results show, that consumers in all four countries perceive PEF and HPP treated juices to be a better choice than pasteurized juice if the price and taste are right, although the acceptance of HPP treated juice was much better, than that of the PEF products. (Olsen et al., 2011)
According to Olsen et al. (2010) familiar technologies are easier to accept than unfamiliar ones like PEF. In case of PEF products information and benefit statement about the technology may reduce the uncertainty associated with the technology and improve its expected liking. Moreover, tasting is affect consumers' acceptance of HPP and PEF-treated products. Hence, experience with the products and more information about the technologies can be the key to achieving consumer acceptance of products made by novel technologies. This is especially important in case of PEF products, since many consumers associated the name of the technology with electricity, and they were sceptical. It is important, that food producers and food scientists must provide the evidence that this technology is safe to use in connection with food processing (Olsen et al, 2010).
Because of the threatening name of PEF Prof. Dietrich Knorr, Chairman of the NovelQ Scientific Advisory Board proposed a new expression „micro pulse" to replace the name Pulsed Electric Field.
In the framework of the present study, our aim was to investigate the acceptance of the new terminology. We examined

- the suitability of the proposed terminology,
- the consumers' concerns about the rename of the technology.

MATERIAL AND METHODS

A qualitative method, focus group interviews, was conducted, to discover consumers' emotions, motivations and attitudes. 6-12 respondents are needed for a focus group, what is moderated by a trained moderator. There is a main topic in the focus of the discussion. Focus groups must have to be homogenous regarding for example the respondents' demographic data, lifestyle or other. The benefit of the focus group interview is the interaction between the respondents within the group, what creates real life situation during the discussion.
In the framework of our research, a preliminary focus group was organized with the participation of 6 respondents. The aim of the preliminary focus group was to design the guide for the consumer focus groups.
For the examination 3 consumer focus groups were organised, altogether with 21 respondents:

- one group with respondents 45 < years (6 respondents),
- one group with young adults 25-40 years (8 respondents),
- one group with health conscious respondents, as the target group of novel products (7 respondents).

The guideline of the consumer focus groups consists of three parts:
1. Review of the discussion objectives in general, introductory question
2. Attitudes toward PEF, 'micro pulse', HPP, pasteurization terminologies

- Associations based on the expression
- Attitudes based on the description of the technologies
- Intention to buy products made of the technologies

3. Opinions, judgements about the alternative name of the PEF technology and the rename of the technology.

Descriptions of the technologies applied in the consumer focus groups, to give information for the respondents. To define the descriptions of the technologies we consulted with Prof. Knorr. The descriptions were short and easily understandable, as product labels in general, as follows:

- **Preserved by pasteurization:** After a short heat treatment the product is quickly cooled down increasing its durability in this way. This process nearly preserves the fresh taste and vitamin content of the product. Refrigerating is required!
- **Preserved by 'micro pulse' treatment:** The product undergoes a short-term electric impulse treatment. Product treated in this way will have longer shelf life in comparison with the pasteurized ones. This process nearly preserves the fresh taste of the product and the heat sensitive vitamin content. The procedure is energy saving, treated products should be refrigerated.
- **Preserved by high pressure treatment:** Durability is provided by applying with extreme high pressure for a short time; shelf life exceeds that of the pasteurized product. This process nearly preserves the fresh taste of the product and the heat sensitive vitamin content. The product should be refrigerated.
- **Preserved by Pulsed Electric Field technology:** The product is given a high-voltage alternating electric field treatment assuring in this way increased durability related to the pasteurized products. This process nearly preserves the fresh taste of the product and its vitamin content. The procedure is energy saving, treated products should be refrigerated.

After the consumer focus groups an **expert group** was organized with 13 experts, to discuss the concerns related to the change of the name of PEF technology. The experts represented the different fields of food sciences: technology, legislation, food policy, consumer sciences, nutrition sciences and decision makers.

A flow chart (Figure 1) shows the procession of the of the focus group interviews.

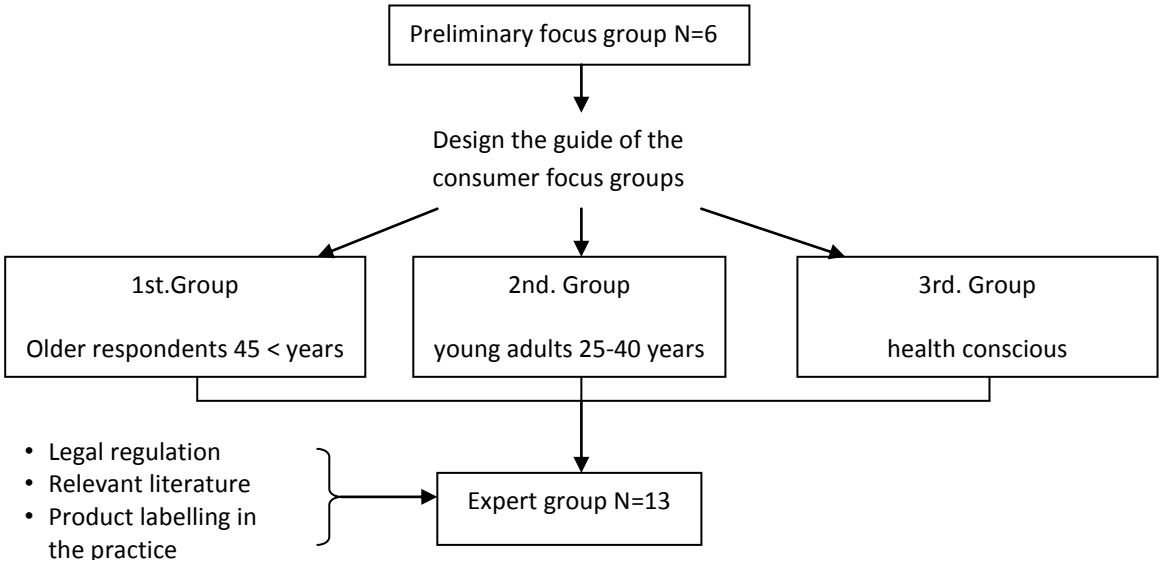

Figure 1.The procession of the focus group interviews

RESULTS AND DISCUSSION

Results of the consumer focus groups

In the first part of the consumer focus groups, associations and feelings evoked by the names of the technologies were collected.

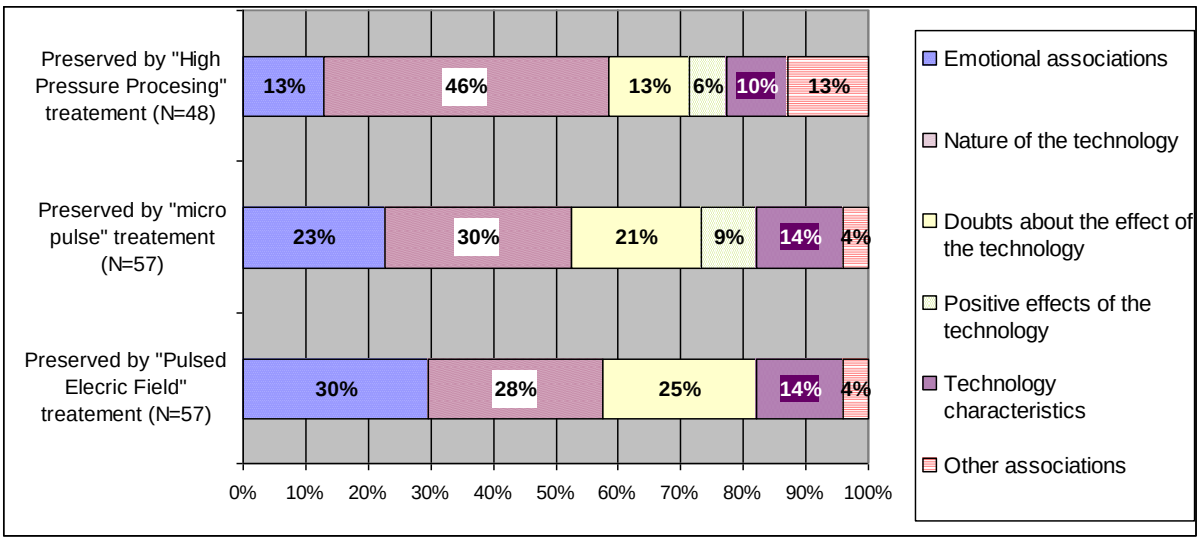

Figure 2. Categories based on associations mentioned (N= number of the mentioned associations)

Figure 2 show the mentioned association categories by the consumers. The most frequent associations mentioned about HPP were related to the nature of the technology (46%), like ‘pressure’, ‘heat treatment’, ‘gas’, ‘vacuum’, ‘homogenization’. Emotional associations ‘higher confidence toward this technology’ and doubts about the technology, like ‘it could be harmful for the nutrients’ were equally frequent mentioned (13%). 10% of the associations were related of the technology characteristics, like ‘simple’ ‘natural’. 6% of the associations related to the technology’s positive effects, like ‘increase the shelf-life”, ‘fresh product’, ‘microbes are destroyed’

More emotional associations (30%) were mentioned regarding the terminology Pulsed Electric Field, than for HPP and these were mostly negative associations, like ‘distrust’, ‘uncertainty’ and ‘fear’. The associations related to the nature of the technology were also negative, like ‘radiation’, ‘roentgen’, ‘electricity’. There were more doubts mentioned (25%) about the effect of the technology, than in case of the other two terminologies (‘unhealthy’, ‘changes in the product’s structure’). Regarding the characteristics of the technology mainly negative associations were mentioned also, like ‘complicated’, ‘expensive’, ‘dangerous’.

In case of the new terminology ‘micro pulse’ more neutral associations were mentioned, than in case of the Pulsed Electric Field technology. The most frequent associations (30%) were related to the nature of the technology, like ‘microwave’, ‘heating’, ‘vibration’. 23% of the mentioning were emotional associations, like ‘inquisitiveness’, ’accepting’, ’uncertainty’. The judgement of the characteristics of the technology was mostly positive or neutral: ‘technology of the future’, ‘small effect’, ‘rapid’, ‘complicated’, ‘expensive’. 9% of the associations were positive associations about the effect of the technology: ‘tactful’, ‘minimal’, ‘less changes in the product’.

To summarize the associations, more positive mentions were related to ‘micro pulse' than to Pulsed Electric Field. The name ‘micro pulse' is in no way associated with 'electricity', but the judgment of HPP was more positive, than that of the ‘micro pulse’ – according to the associations and the descriptions, too.

Young consumers are more open toward the novel technologies, while elder consumers usually more conservative. The group of health conscious consumers was the most risk sensitive – although they are the target group of the novel processed products. On the other hand, the avoidance of food additives is a benefit for them.

Consumers in all groups expressed their definite need to be informed about the technologies, even if this leads to uncertainty and distrust because of the eventual lack of comprehensive knowledge.

Consumers’ opinion about the name changing is divided:

According to two focus groups, it would not be misleading to rename PEF into ‘micro pulse’, though the electrical feature of the treatment should be mentioned.

According to the respondents of the health conscious focus group, changing the name of the technology is misleading, because the aim is the reduction of consumers' fears. A deterrent technology name makes consumers more careful and motivates more conscious product choice.

Main results of the expert focus group

According to the experts of food sciences, the description of the technology should be explained to consumers on the product label, or with the help of other communication tools. Clear and easily understandable communication about the novel technologies is very important. The electrical nature of the technology might have been communicated, because consumers expressed their need to be informed. The name ‘pulsed electric field’ is threatening the term ‘micro pulse’ is not informative. The terminology ‘micro electric pulse’ is acceptable.

Novel technologies have to offer unambiguous benefits to the consumers what is facilitating the marketability of the novel processed products.

The product label is not enough in itself for adequate information providing. A communication campaign is needed in order to introduce novel technologies: besides the product labels the use of other communication tools is needed.

Communication between the experts and the opponents of the technology might be an efficient solution to release doubts related the novel technologies.

CONCLUSIONS

In case of the market introduction of a novel food technology, consumers’ conservatism should be taken into account. The main problem is that although consumers need information it is difficult to explain clearly the nature of the technology clearly.

Consumers expressed their definite need of getting informed about the technologies even if this leads to uncertainty and distrust. Clear communication is important for consumers; the description of the technology should be explained for them.

The term 'micro pulse' creates better associations than pulsed electric field and 'micro pulse' is in no way associated with 'electricity'. However, according to the respondents of the health conscious focus group (the target group of the novel technologies), changing the name of the technology is misleading. The name ‘Pulsed Electric Field’ is deterrent and ‘micro pulse’ is not enough informative. The name ‘micro electrical pulse’ would be acceptable, according to experts.

It is essential for the marketability of a novel technology that it has to offer unambiguous benefits for consumers. Besides the information provided via the product label a communicational campaign is needed in order to introduce novel technologies.

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VISCOELASTIC STUDIES OF FRESH MANGO PUREE TEXTURISED WITH GELLAN GUM

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ABSTRACT: Consumption of fresh fruit products increased during the last decade, probably as a consequence of the public awareness regarding the importance of healthy eating habits and consumer's tendency for eating ready-to-eat fresh cut fruits (Olivas G.I., et al. 2005).
The objective of this study was to develop a jellified fruit product based on mango puree and gellan gum. The effect of type of gellan (low acyl and high acyl), concentration (1% and 2%) and dissolution temperature (70 ºC and 90 ºC), on gels setting temperature, was investigated.
Gels prepared with mango puree and low acyl gellan have shown higher values of dynamic moduli than those with high acyl gellan. The heating temperature was determinant on the physical properties of the mango puree-gellan: gels produced after heating the mixtures to 90 ºC for 1 min have shown higher dynamic moduli and higher gel setting temperature (Tg≈ 65 ºC), than those obtained after heating at 70 ºC for 1 min (Tg≈ 45 ºC).
Future work will be focused on the optimisation of the product formulation (e.g. use of low acyl and high acyl in different proportions) and production process, in order to design a fresh fruit product with an improved sensorial acceptance.
Key words: *jellified mango puree, gellan gum, viscoelastic properties*

INTRODUCTION

Ready to eat fresh fruit products became an important area in the food industry due to their characteristics of freshness, low caloric content and convenience, enabling their use in the promotion of fruit as a basic component of a healthy diet (Corbo et al., 2000).
Gellan gum is an approved gelling agent, available in two forms with different characteristics (low acyl-LA and high acyl-HA), having interesting properties that are applicable to a wide variety of food systems. It is an extracellular polysaccharide secreted by the bacterium *Pseudomonas elodea,* which can produce a gel at a concentration as low as 0.2% in the presence of cations. The gellan gum structure is based on a tetrasaccharide repeating unit composed of (1-3)-β-D-glucose, (1-4)-β-D-glucuronic acid, (1-4)-β-D-glucose, and (1-4)-α-L-rhamnose as the backbone (Noda et al., 2008). The gelation process is generally considered to involve two separate thermo-reversible steps. Molecules of the gellan gum adopt a disordered coil (single chain) upon heating in aqueous solutions. Upon cooling the molecules adopt an ordered double helical conformation followed by associations between the helices through weak interactions such as hydrogen bonds and van der Waals forces (Matsukawa et al., 2007).
Its multi-functional properties, its synergy with other polysaccharides and between the two gellan forms (LA/HA), allows the production of a wide range of textures, which make gellan gum an attractive additive to be used in the food industry (Chandrasekaran et al., 1995).
Tropical fruits, particularly mango (*Mangifera indica L*.), have a high content of antioxidant compounds, including carotenoids, vitamin C, vitamin E and phenolic compounds (Vijaya Kumar et al., 2010; Xianli et al., 2004). Thus, the consumption of mango may provide significant amounts of bioactive compounds with antioxidant activity. Due to its pleasant

colour, taste, good texture, and flavour, mango attracted attention for minimal processing as a fresh-cut product (Gonsález-Aguilar et al., 2008).
The interactions between gellan gum and mango puree depend on their physical-chemical properties and on processing conditions, such as temperature, mango puree/gellan proportion, ions concentration and pH. To have a better understanding regarding gelation mechanism and physical properties of mango puree with gellan gum, the effect of type of gellan (low acyl-LA or high acyl-HA), LA/HA mixture (50/50) and gellan dissolution temperature (70 ºC and 90 ºC), on the gel-setting temperature and viscoelastic properties, was studied.

MATERIAL AND METHODS

Materials

Low-acyl gellan gum (Kelcogel® F) and High-acyl gellan gum (Kelcogel® LT) were provided by CP Kelco U.S., Inc. (Wilmington, DE, USA).
Mature mangoes (Mangifera indica L. cv. Palmer) were purchased from a local supermarket in Lisbon, Portugal.

Preparation of the samples

Before processing, mango was stored at 4±1 ºC. The fruit was washed, peeled, cut into small pieces, and pureed in a food blender for 10 minutes. The puree obtained was heat treated for 5 minutes at 70 ºC, in order to induce the enzyme deactivation. pH was measured using a pH meter calibrated with standard buffers. The total concentration of soluble solids (TSS) was determined with a digital refractometer PAL-1, calibrated with distilled water.
Gellan gum (1%wt and 2%wt) was dispersed in the mango puree by stirring and adding the powder slowly to the vortex. Samples were also prepared with 50/50 LA/HA proportion at overall polymer concentration of 1%wt and 2%wt.

Dynamic oscillatory measurements

Rheological measurements were performed on a controlled Stress Rheometer, RS 75 Rheostress HAAKE. The sample was placed between two parallel stainless steel serrated plates (gap of 1 mm), with a diameter of 35 mm. Paraffin oil was applied along the border of the plates to prevent sample dehydration.
The experimental procedure consisted on 2 steps : (i) heating at 90 ºC (or 70 ºC) and holding at that temperature for 1 min, (ii) oscillatory cooling temperature sweep to 20 ºC at a rate of -2 ºC/min (f = 0.1 *Hz*, τ = 1 *Pa*).

RESULTS AND DISCUSSION

Fig.1 shows the gelation profile of mango puree/gellan mixtures upon cooling. When Low Acyl Gellan was used (Fig. 1a and 1b) a rise of G' and G'' was observed, which was attributed to gel setting process. However, the gel setting temperature (Tg) was dependent on the temperature at which the mixture was heated. Mango puree/gellan mixtures heated at 90 ºC show a Tg around 65 ºC upon cooling, while a Tg≈45 ºC is observed when the mixture was heated at 70 ºC. In addition, stronger structures were obtained upon cooling from T=90 ºC, since higher values of dynamic moduli were reached in this case. It may also observed that, the increase of Low Acyl Gellan content from 1% (Fig 1a) to 2% (Fig 1b), does not induce an improvement in gel structure formation.

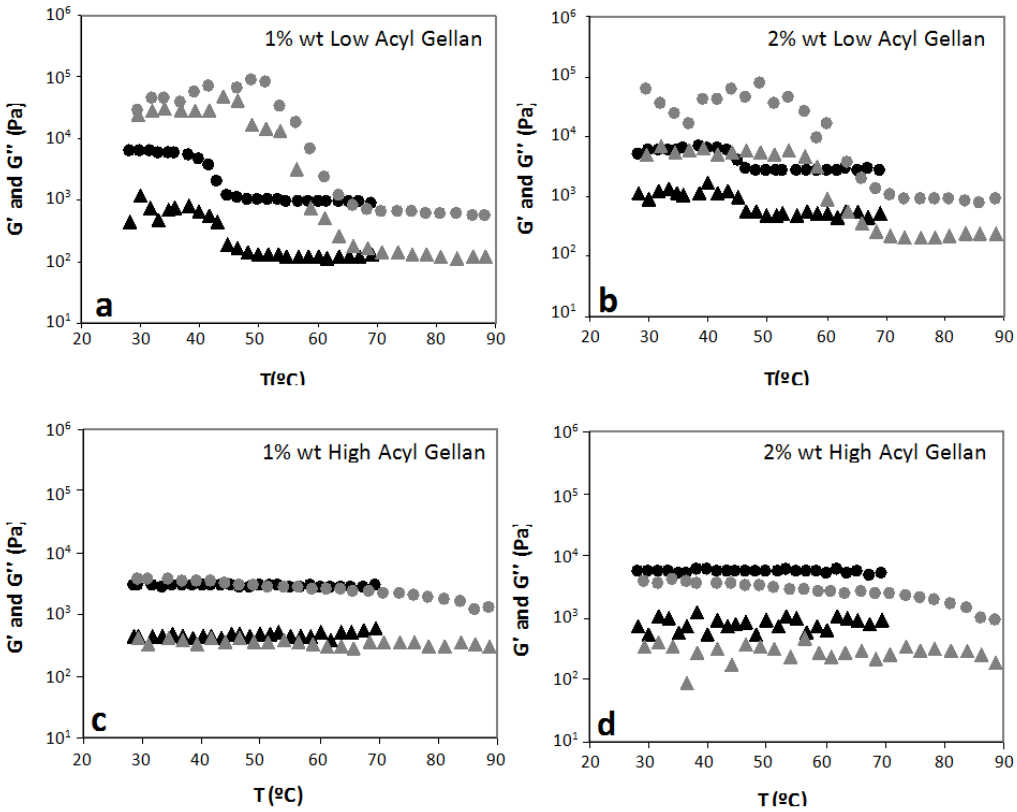

Figure 1: Dynamic moduli of gellan gum/mango puree mixtures, G' (●, ●) and G'' (▲, ▲), as a function of temperature during cooling from 90ºC (gray symbols) and from 70ºC (black symbols).

When High Acyl Gellan was used as jellifying agent, no marked increase on dynamic moduli was noticed over the temperature sweep (Fig. 1c and 1d). Furthermore, High Acyl Gellan has shown to be less effective on structure creation, as in the end of the temperature sweep the values of G' and G'' were much lower when compared to those obtained with Low Acyl Gellan.

The formulations having LA/HA (50/50) mixtures, have shown a gelation profile similar to the one observed only with Low Acyl Gellan (Fig.2). Low Acyl Gellan is known to produce more firm and brittle gels, while the texture of high acyl gels is more soft and flexible (Philips, G., 2000). It is expected to obtain texturized mango puree with intermediate properties by using gellan mixtures.

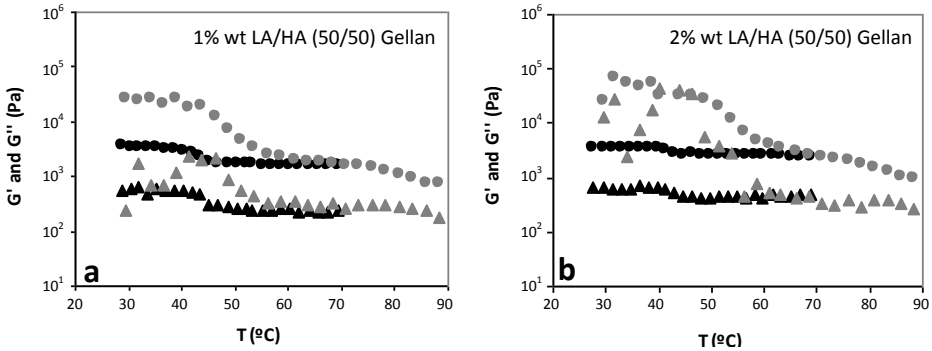

Figure 2: Dynamic moduli, G' (●, ●) and G'' (▲, ▲), of mango puree texturized with HA/LA (50:50) mixtures, during cooling from 90ºC (gray symbols) and from 70ºC (black symbols).

CONCLUSIONS

The effect of type of gellan (low acyl-LA or high acyl-HA) and LA/HA gellan mixtures, on the gel properties was studied using a stress controlled rheometer which monitored the variation of G' and G'' under cooling temperature sweeps. It was observed that: (i) Low Acyl Gellan has shown to be more effective as texturizing agent of mango puree than High Acyl Gellan, (ii) the increase of gellan content from 1% to 2%, does not induce any improvement in gel structure formation, (iii) the heating temperature was determinant on the physical properties of the mango puree-gellan structure: gels produced with Low Acyl Gellan after heating the mixtures at 90 ºC have shown higher dynamic moduli and higher gel setting temperature, than those obtained after heating at 70 ºC, (iv) the formulations having LA/HA (50/50) mixtures have shown a gelation profile similar to the one observed only with Low Acyl Gellan.

ACKNOWLEDGEMENTS

This paper is a result of the research within the Grant (SFRH/BD/64341/2009), financed by Fundação para a Ciência e a Tecnologia. The authors thank to CP Kelco company for providing the gellan.

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WHAT DO YOUNG PEOPLE THINK ABOUT FOOD RELATED INFORMATION ON THE INTERNET?

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ABSTRACT: The aim of this study was to investigate if young people, and in what way and to what extent, use the Internet as the source of information about food, its composition and dietary habits. The sample consisted of students of Faculty of Economics University of Mostar (BiH). Young people use the Internet very often, mostly up to 3 hours per day. They read news, play games and socialize on social networks. A lot of respondents consider that food and dietary habits affects human health. Most of them talk about food with family and friends, but very few of them read the laws on food and food products. Students think that there is enough information about the food available on the Internet. A small number of respondents use Internet to find out something about food, and could specify the web site with the theme of food or purchase food through the Internet. The results show that searching the Internet with the aim of discovering new knowledge about food and its beneficial impact on human health is not sufficiently affirmed among young people and the environment should work to encourage the aforementioned.
Key words: *Internet, food ingredients, young people, dietary habits*

INTRODUCTION

The food is the „fuel“ of the people and has significant impact on the human health. Therefore, it is necessary to be familiar with the quality and the composition of the food products. Primarily source of the information about the composition of the food are the declarations on the food package, but this is rapidly changing, due to the development of the new media. Fast development at the area of information and communication technologies led to the fact that in the last 20 years the Internet has become a new important source of the information for the customers, so some, before unimaginable and incomprehensible, information have became readily available and known.

Internet is an interesting phenomenon and people approach it in different ways. For some, it's the place of many information, news and knowledge, for others, it's the place of pure fun and amusement, yet for others it's the place of making business and earning money and even for some it's the place of bad or evil behavior. From the social point of view, Internet is becoming essential part of the everyday life whether it's used during free of working time. It's inexhaustible source of information from and for all areas of human life which makes it a good foundation for various research about the frequency, purpose and benefit of its usage.

At the review of the available materials, not many researches connecting food and the Internet were found. There are many papers that research the consumer awareness about the nutrition data at the packaging of food products (Sharf et al., 2012), how consumers assess the product based on logo (Hoogland et al., 2007), the impact of nutrition data available on the product to the relation of consumer and healthy diet (Barreiro-Hurlé et al., 2010) and the importance of the standardization of the information at food and its availability to every consumer in the world (Bell et al., 2011).

Bearing in mind the importance of the awareness about the consumed food, and under the influence of the information-communication technologies, it’s not possible not to wonder if the Internet is used for the purpose of spreading the knowledge about food and food products. This issue was the foundation for defining the aim of the conducted research.

The aim of this study was to investigate if young people, and in what way and to what extent, use the Internet as the source of information about food, its composition and dietary habits.

MATERIAL AND METHODS

The survey was conducted among students of the Faculty of Economics, University of Mostar in November 2011. A random sample included 140 respondents in all years of study (from first to fifth), with age range 19 – 24. Before implementing the survey, students were informed about the object and purpose of the research. The survey process lasted 15 minutes. After the technical and logical control of the questionnaires, 132 of them were accepted for further analysis. Questionnaire was used as the survey instrument. It contained questions about the purpose and frequency of Internet usage, attitudes about food, and the usage of the Internet as a source of information on food products. Questions were formed as closed form questions, allowing respondents to answer by choosing one or more answers.
The results are expressed in relative frequencies (one decimal place). SPSS version 12.0. was used for statistical analysis.

RESULTS AND DISCUSSION

The survey included 132 respondents, 42.4% men and 57.6% women. 88.6% of the respondents live in their town of study, and 69% of them live with their parents and others in student dormitory or private housing. The rest of the students travel daily to their faculty by personal car or bus, and they mostly live with their family or relatives.

Internet

- Nearly 80% of the respondents use the Internet daily (78.8%), 18.2% do so several times a week, while other 4 respondents stated that they do it even rarer.
- For the majority of the respondents one Internet session lasts 1-3 hours (47.7%), 27.3% of them finish all their activities within one hour, while 6.1% of respondents are almost dependent to the Internet, spending more than 5 hours in one session. Other respondents use the Internet in the continuum between 3 and 5 hours.
- Half of the respondents believe that only 25% to 50% of the time they spend on the Internet is useful for them, 25% believe that the usefulness percentage is less than 25% and only 5.3% of respondents considered that all the time spent on the Internet is useful (100% efficiency).
- Most of the respondents only partially believe the information available on the Internet, (70.5%), while only 5.3% of respondents stated that they believed completely. Other respondents take all of the available information with caution.
- 58.3% of the respondents had at least once checked the accuracy of the information found on the Internet, while the others never did so.
- The most common activities on the Internet are: socialization at social networks, playing games, watching movies, listening to the music...

Food products

- Preferences of the students by type of food they consume are usually quite colorful. The proposed responses are represented as follows: healthy foods - 32.6% of the respondents, junk/fast food - 21.2% of the respondents, traditional food - 28% of the respondents. The remaining 18.2% of the respondents stated that they eat something else; thereby indicating the combination of previously mentioned three categories or the indecision to declare what they prefer.
- With regard to the criteria of choice of food/food products, students mostly go for flavor (66.7%), followed by the composition of ingredients (20.5%) while the price and packaging are poorly represented.
- Slightly more than half of the respondents consider their eating habits to be average (neither good nor bad - about 55%), about 30% thought that their eating habits were very good or excellent, while the rest of them find their eating habits more bad than good. None of

the respondents choose the answer “bad”, while 5 respondents felt that their eating habits were excellent.

- 99.2% of respondents believe that food can affect health, and 96.2% consider this to be the case for diet regime.
- 92.4% of the respondents consider that it is important to be well informed about the composition (ingredients) of the food products, but they are less interested in the composition of the food products they consume (68.9%). 72.7% of the respondents read the composition of food they consume, and 75.8% of them talk about it with people from their environment. Only 14.4% of the respondents read the applicable state laws related to food products.

Internet & food products

- Slightly more than half of the respondents (56.7%) stated that they at least once searched the Internet to obtain new information about food, or to put it differently: they were in a situation where they heard something new about the food and they used the Internet in order to know the importance of the new terms.
- Nearly ¾ of respondents believe that the Internet is a good source for the information related to food in general, food products and diet.
- Less than half of respondents use the Internet to gather information about food in general (40.9%) or specific food products (42.4%).
- When it comes to the information about food and food products that respondents search the Internet for, it turns out that it mostly comes down to recipes (instructions) for the meal preparation (61.4%), followed by information on the impact of food on health and which food is good for what (at 48% of respondents). Method of preparation of certain foods is a topic of research on the Internet for 37.1% of the respondents, 20% and 25% of them search the safety and composition of food products respectively, 24.2% of them compare people's experiences, while the method of storage and process of manufacture of food products are interesting to less than 10% of respondents.
- The results showed that most respondents only partially believe the information from the Internet (67.4%), 25.8% do not believe in the information at all, and only 6.8% of the respondents consider that the information found on the Internet is completely accurate.
- Even though respondents distrust the information on food available on the Internet, only 32.6% respondents check the accuracy of the same.
- Very few respondents use the Internet as a communication media that is to exchange information about food and food products with others. 12.9% of the respondents confirmed that they receive various and useful food related e-mails from their friends, and 10.6% were forwarding these e-mails to others so that can get familiar with the benefits.
- When asked if they knew and could specify the address of a Web site whose primary theme is the food a few respondents indicated www.coolinarka.com page. If the collection of information about food can imply different cooking recipes then the study found that 61.4% of respondents use the Internet for these purposes. Only 7.6% of respondents, namely only 10 of them, are included in certain groups on the Internet that deal with the theme of food.

DISCUSSION

What is the reason for using students for the research, especially because they're such young population? The reason is because young population is most frequent and most common consumer of the Internet when it’s used as the communication media and source of information.

The research results of the students’ Internet habits have demonstrated that Internet is very present among them and that, in most cases; they spend around 3 hours per day browsing. The most common activities that they get involved in when using Internet are socializing in social networks, reading news, playing games and exploring of different subject areas that are of great interest to them. Most respondents feel that even though they use lot of time at the Internet, that time is not well spent. Besides, Internet is an inexhaustible source of the information that respondents use in everyday life, but on the other hand they are familiar with the fact how quality control of available information is not fully guaranteed and so they take

Internet information, which they operate with, with caution (as being partially correct), and accordingly they are familiar with the activities of verification of gathered information.
Analysis of issues related to the eating habits of students has produced a lot of colorful responses. All offered types of foods are equally represented, and it is not possible to identify student population with just one kind of food. It was expected that students would be choosing an answer junk/fast food in more cases, due to the strengthen influence of the western culture, but this was not the case and the results negated the presumption. The reason for this can be found in the fact that most students live with their parents in their place of study and due to the local tradition they are guaranteed with daily homemade (cooked) meal. Despite low percentage of students who prefer a healthy diet, almost all respondents believe that food and diet affect health, which is positive, but is in contrast to their criteria of selection of food (taste). Why is this in contrast, it's because "unfortunately" ingrained notion that healthy food is tasteless. Further on, young people believe that one should be informed about food, but the results suggest that very few of them read food composition of ingredients, legislation or even discuss the topic of food with others.
As the research showed, Internet is still a place of fun for young people, and not the primary source of information. Actually, for them it is the source of information, but information that are usually not important for the daily functioning of the organism. The Internet is just slightly presented as the source of information about food, food products and diet even though young people consider that it offers enough of such information. The question that arises here is: On what basis does youth form such attitude. It can be assumed that the answer is: logical thinking. Since the Internet abounds with "all and everything" it can be assumed that enough information about food products can be also found and thus: the students' opinion. In fact young people right about it, the Internet abounds with information related to food, but they are also unmistaken for taking that information with doubt, not trusting it completely. Abovementioned attitude towards quality of information is correct and further analysis should investigate the quality of information available about food, with special emphasis on the reasons for their presentation on the Internet. The reason for this skepticism lies in the fact that nowadays the Internet is the mean of communication with interested parties, in most cases with the primary aim of making profit. This applies primarily to the economic sector, because when the results of the conducted research are being analyzed in respect to the Internet as a communication medium, they are not so attractive because very few respondents states that the Internet is used for the exchange of information about the food with their environment.
Neither familiarity of respondents with existing web pages that deal with food issues has shown interesting results. Specifically, since asked to name a web page with the theme of food, they mostly stated www.coolinarka.com, and bearing in mind that the primary purpose of this site is sharing recipes for the preparation of various foods, it can be concluded that food web pages are considerable unknowns to them.
Despite the frequent use of the Internet it turns out that respondents do not think about purchasing some groceries via the Internet (online shopping), which coincides with their unfamiliarity and under-utilization of the Internet as an information resource. The reason for the above lies in the fact that electronic commerce in BiH has not been realized in full swing, it's still at the stage of growth and great distrust of the consumers for the mentioned form of trade is present. Perhaps it's a little strange to discuss the purchase of food through the internet, but sometimes it is simply necessary, especially in the case of territorial limits for production of some foods. On the other hand, however, the increasing number of Internet users leads to "internetisation" of all daily activities and most of the big shopping malls that are present on the Internet offer online shopping options. Consumer purchases in the virtual store, and after a period of time, all purchased goods get delivered to his/her address. These services every day stronger and the online food shopping will soon be something we take for granted, and soon, thanks to lifestyle and practice for many. These services are getting stronger by every day and the online food shopping will soon be something we take for granted, and thanks to fast lifestyle also a common practice for many.

Results showed that very few respondents used thematic website (this refers to sites which don't have sales and promotion of some products as their primary goal, but education, or approaching some unknown facts to the common man) and forums for exchanging their personal experiences and discussion.
Given the above facts it is easy to conclude that the segment of young consumers is most convenient for the promotion of food through the Internet, whether it is promoted with the goal of increasing sales (as viewed by the company) or the goal of encouraging healthy lifestyle.

CONCLUSIONS

The obtained results indicate that education on Internet possibilities should be strengthen, and quality of information on the Internet put in focus in order for young people to acquire habits of Internet search and of checking the available information since it is obvious that the frequency and degree of implementation of the Internet in everyday life will continue to grow with no clear indication of the opposite.
Given the fact that young people are aware of the positive impact of food, intensifying the promotion of healthy food should be worked on, and student ways of Internet usage should be utilized as the basis for all promotional activities to promote awareness of healthy eating and essential knowledge of food products of, but also to promote the positive characteristics of the Internet in general.
In order to intensify the usage of the Internet for "health" causes, it would be very convenient and educational to organize forums and workshops at which young people could get acquainted with ways to search online information and ways to assess its quality, accuracy, authenticity and acceptability. These events should be organized with joint efforts of student organizations, doctors, nutritionists and course teachers (and teaching assistants) who are specialized in the field of Internet, electronic business, food and its effect on human. Synergy effect of all mentioned areas of expertise would help create appropriate image of Internet-food correlation and its meaning for healthier and better quality life in the minds of young people. All of this could lead to even more detail understanding of student habits and could trigger them to share this newfound, interesting and useful information through e-mail, but also through online forums or social networks which are, according to current research, unavoidable accessory to personal and business life.
The limiting factor of this study is the fact that these are the students of economic faculty. Continuing the above mentioned research would involve the participation of students from other faculties, especially the Agriculture and Food Technology and Biotechnology Faculty in order to analyze the intensity and frequency of their Internet usage. This could then be compared with other students in order to draw conclusions about the possible impact of study area to the use of the Internet for research in the area of food and food products.
In addition, research should be enhanced with questions about socio-demographic and health features followed by comparison of different sub-samples. Further on, analysis of web sites, which are focused on food, should be conducted, analyzing the quality of available information and the quality of web pages themselves.
This and future, as recommended, researches would create a good foundation for the creation of appropriate strategies of action in an environment that includes young people, Internet (technology) and food as the driving force behind it all.

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WHY DO WE NEED TO CONDUCT SENSORY TESTS ON CONSUMERS? THE CASE OF INNOVATIVE SWEET TOPPING

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ABSTRACT: A significant component of new food development process is precise identification of consumer preferences and expectations. In this paper we empirically show that findings form a preliminary research, can be used for development guidance, however, when more objective research techniques are applied, suggested managerial decision could critically differ. The new food product is dessert topping based on traditional Slovenian wine teran, which is highly valued among consumers and perceived as a national speciality. We assumed that these positive elements of consumers' perception about wine teran will be transferred to new developed product and will form central marketing elements.
The research involving consumers combined semi-structured interviews, focus groups and questionnaire with consumer sensory test. The third phase aimed at product fine-tuning, applying consumers' sensory tests; hedonic evaluation and preference testing, where four commercial toppings were included besides the newly designed one. Questionnaire gave important inputs to the marketing strategy, elucidating the purchasing behaviour and consumer expectations regarding the products in target market segment.
We found out that the market is in highly mature phase. As distinguished from the quantitative stages of the research, the questionnaire reviled consumers' preference for established topping flavours, particularly chocolate. Consumer sensory test disconfirmed high expectation, since the average score in preference testing was in lower end for teran's topping, being surpassed by the standard topping tastes. Market acceptance of the new product is below the threshold to continue the outlined product development strategy without risking business failure that is why we suggest product concept redefinition.
Key words: *new food product development, consumer sensory testing, preference test, hedonic test, prototype optimization*

INTRODUCTION

New food product prototypes that include consumer needs and wants have a greater chance for success on the market. However, in reality smaller food companies often neglect that phase and launch the new products without detailed market and consumer analysis. This specific research field stresses the necessity to attack problems in an interdisciplinary way, which is still rare in both sensory and consumer research (Köster, 2009).
Successful new products are usually result of comprehensive market research, planned product development program and an organized marketing effort (Side, 2002). Consumers want to be able to choose the products that consistently meet their needs and desires (Perry and Cochet, 2009). It is a generally accepted fact that far too many food product introductions fail (Costa and Jongen, 2006; Linnemann et al., 2006). Side, 2002 estimated a failure rate of over 90%. The majority of innovations (approximately 65%) are line-extensions aiming at variation (Linnemann et al., 2006). According to Lord (2000), 72% of true new products and 55% of line extensions fail. For non-leading, smaller companies a success rate is only 12% (Lord, 2000). Most part of the food industry has relied on an incomplete knowledge of consumer preferences (Moskowitz and Hartmann, 2008).

We noticed that Slovenian food companies in last few years didn't show significant innovative progress. Slovenian food industry is one of the most important manufacturing sectors and lack of innovation and new product development can have significant effects on the market position of Slovenian companies. One of the major deficits is certainly a decline in industrial research and development processes, which includes the development of new products. To ensure success in developing new food products is necessary to include, in early stages, findings obtained with different consumer analysis techniques. Consumer sensory testing of prototypes is a method which allows the optimization of a new product and ensures a greater chance of commercial success.

Sensory characteristics should not be overlooked, because they almost always have a significant weight in the final decision (Combris et al., 2009), particularly in the new product development, much of the success or failure of a food product in the marketplace results from consumer perception of sensory quality (Moskowitz et al., 2006). Sensory test on consumers attempt to quantify the degree of liking or disliking of a product, called hedonic or affective test methods (Lawless and Heymann, 1998). In studies about food acceptability, four critical questions arise: how consumers perceive the sensory characteristics of food; to what extent the variation in perceived sensory characteristics influences consumer response; how certain consumer habits, attitudes, or beliefs affect hedonic ratings and purchase intention and to what extent hedonic ratings are influenced by the expectations created by different types of information (Costell et al., 2009). Innovation for mass markets is characterized by the lack of personal interaction between the innovator and the users or, at least, most users (Grunert et al., 2008). Sensory evaluation and market research are two separate but related activities that complement one another and rely on different testing methods (Resurreccion, 1998). The primary role of sensory analysis is to provide information about how the sensory characteristics of products, ingredients, or other related information and services relate to perceived quality characteristics and to consumer liking. Once the quality attributes important to the consumer have been determined, appropriate design for systematic product optimization can be developed (Moskowitz et al., 2006).

There are two approaches to consumer sensory testing, first is acceptance test, which is a valuable and necessary component of every sensory evaluation program; it measures consumer's acceptance or liking of a product (Moskowitz et al., 2006). In contrast, preference tests measure the appeal of one food or food product over another (Stone and Sidel, 1993). The consumer panel gives opinions on all product characteristics, not just sensory qualities but others such as safety, nutrition, size, ease of use, transport, storing and convenience. They can also be involved in the design of the package. (Earle and Earle, 2000).

New food product is dessert topping made from traditional Slovenian wine, which is highly valued among consumers and perceived as a national speciality. We wanted to see consumer response to the new product and test the market potential. Research was carried out in three stages: semi-structured interviews, focus groups and a questionnaire with consumer sensory test.

MATERIAL AND METHODS

First qualitative phase – interviews and focus groups

The most popular qualitative research methods for the design stage are focus groups and in-depth interviews (Costa, 2003), they are good tool for the critical properties of the product, which can be modified and adapted during process of development (Resurreccion, 1998). Qualitative methods are ideal in the early fuzzy-front-end of the innovation process, where the objective is, for example, to gain a deeper understanding of user needs (manifest or latent) in order to be able to formulate a product concept, but knowledge about consumers' experience, their beliefs and understanding may also be valuable in later phases of product development (Grunert et al., 2011). They generate oral-descriptive, non-numerical information, and are usually carried out within small groups of people (Costell et al., 2009).

In our research we used semi-structured interviews, where questions are predefined, but we can adapt flow of conversation to the situation. The advantages of in-depth and detailed

responses can be queried for, which may provide the researcher with new insights and ideas for the new product development process (Kleef et al., 2005). We interviewed twenty consumers, male and female, different statuses and age groups on a fair in Ljubljana. Next step were two focus groups, where 14 consumers were involved. The function of the focus group may be to suggest ideas, to clarify potential options, to react to ideas, to recommend a course of action, to make a decision, to plan or to evaluate (Krueger and Casey, 2000).

Second quantitative phase – questionnaire and consumer sensory

The third phase was therefore aimed at product fine-tuning applying consumer sensory tests and to get the further insight for marketing and distribution strategy. Sensory analysis was conducted in three different locations in Slovenia and it based on 126 respondents. With obtained data we studied consumer behavior, preferences and expectations about desert toppings. Two sensory techniques were used; namely preference testing and hedonic evaluation. In preference test we compered newly formed teran's dessert topping with four commercial toppings that are available on the market. We used traditional 9-point hedonic scale, which is performing equally well as comparable scales (Lawless et al., 2010). Methods that include a set of competing alternatives available in the market have the advantage, because they represent the task that consumers typically perform in the market (Kleef et al., 2005). Part of the hedonic test was JAR (just about right) scale for evaluating the degree of liking for sweetness, colour and thickness of the new product.

RESULTS AND DISCUSSION

Results gained with interviews and focus groups were formed into specific categories; the main findings are written and shown in table 1. Conclusions from the first two stages gave us qualitative data which was highly encouraging, since the response of the consumers was supportive for the product concept and the expected market potentials were substantial.

Table 1. Results of a qualitative research methods, semi-structured interviews and focus groups

Aspect	Meanings	Aspect	Meanings
Desserts	Associating desserts with different occasions (after lunch, celebration, snack in front of a TV…). Different motives for desserts consumption (happiness, award, sadness…). Lack of time – ready to eat desserts. Desserts prepared home differ on season.	Wine teran	Good, red wine with rich flavour Slovenian, traditional, highly valued. Associating with seaside.
		Packaging for new topping	Natural, ecological. Small, convenient. Material – glass.
Dessert toppings	Used very rarely, few times per year. Unfamiliarity of the dessert toppings available on the market. Lot of ideas for combining topping with different dishes. Curious to try new topping flavor.	Description of the taste	Good impression. Pleasant unknown taste. Intense colour. Presence of alcohol is not distracting.

The statistical analysis of the questionnaire was used to determine consumer habits regarding food, desserts and dessert toppings. Almost three-quarters of respondents want to test new foods that they do not know. Another important factor when choosing a food is the Slovenian origin, since two thirds of the respondents would chose food from Slovenian companies, if this is possible. In turn, the results of the real food choice experiment revealed that activating domestic origin cognitively actually results in favouring foreign food products while activating domestic origin affectively leads to favouring of domestic food products (Luomala, 2007). Concern for the body weight is not the most important factor when choosing food. Only thirty per cents of the respondents fully or partially pay attention on their weight. More than half of respondents have no bad conscious after eating a dessert.

Consumers who participated, eat dessert toppings very rarely; half of them only few times per year. One fourth of respondents are regular users, which consume dessert toppings several times a month and the other quarter almost never eat dessert toppings (less than once a year). The most common reason to not consume dessert toppings is that they don't remember to buy it and use it and also high additive content and high calorific value. They think that there are not enough offers in the market.

Extrinsic factors, like packaging material, commonly have no direct influence on the characteristics of the product, but they can be of overriding importance in the purchasing policy of some consumers (Linnemann et al., 2006). Most of the respondents recommended glass packaging.

Results of preference test are showed in figure 1. Highest average of scores received chocolate dessert topping (6.86) and the new teran's topping (5.28) reached fourth place.

All results have a high standard deviation, which shows the dispersion of ratings on a large scale around the arithmetic mean. The maximum standard deviation (2.65) has teran's dessert topping, as shares of scores are very evenly distributed.

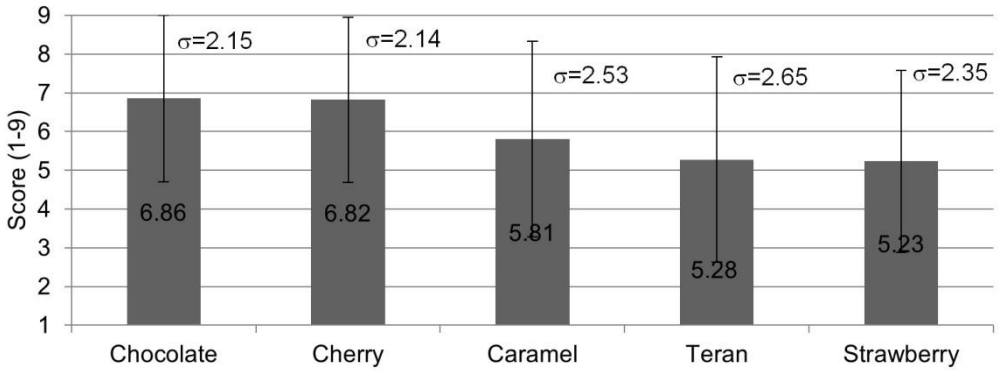

Figure 1. Results from preference testing

In hedonic test teran's dessert topping scored higher average (5.95) than in preference testing. We assumed that could occur because of the inscription "new" in the hedonic test, which lead to a psychological, leniency error. That is when panellists rate the product on the basis of their feelings toward the group or individual that developed the product (Resurreccion, 1998). Particularly increasing was the percentage of best estimates. Sensory attributes from JAR (just about right) scale showed results that were rather favorable for the new product. Product could be better adapted to consumer preferences if it would be a little thicker.

We made three categories of consumers depending on score they gave to teran's topping in hedonic test: enthusiastic, undecided and unfavorable. A variable may be categorical because categories are investigated, but may also have been made categorical by splitting a continuous axis up into subgroups (Næs et al., 2010). With contingency tables and chi-square test we compared and analyze the relation between two categorical variables. In table 2 are chi-square values from comparing hedonic scores with scores of some statements which show statistical significance ($p<0.05$).

Table 2. Comparison of hedonic test scores with certain statements scores that show statistical significance ($p<0.05$)

Hedonic test score	Chi square value	Statistical significance
It's fun to try new foods.	10.608	0.031
If it's possible I choose food from Slovenian companies.	9.963	0.041
I don't have bad conscience when eating a dessert.	10.274	0.036

There is a statistical significant (0.031) connection between degree of liking of new dessert topping and degree of agreement with a statement which indicates neophilia (affinity for novelty). There is a statistical significant result when comparing hedonic scores to degree of loyalty to Slovenian food industry and degree of liking sweets in general.

CONCLUSIONS

Findings form a preliminary research, can be used for development guidance, however, when more objective research techniques are applied, suggested managerial decision could critically differ.

A shortcoming of in-depth and detailed responses research is that the in-depth and idiosyncratic information obtained does not lend itself for direct use in subsequent analysis. A categorization and quantification step is required on the basis of subjective interpretation on the part of the researcher. As such, the personal view of the researcher may affect the way the data are interpreted and a researcher bias can occur as a result from selective observation and recording of information (Kleef, 2006).

The respondents liked the new product, but they preferred the classic. The final results indicate that the market acceptance of the new product is below the threshold to continue the outlined product development strategy without risking business failure. Therefore the process of the product concept redefinition should be initiated in order to better respond to the specific consumer segment or marketing channel. It was find out that the market is in rather highly mature phase with consumers behaving primarily habitually with low involvement in purchasing decision process. Despite the clearly demonstrated inclination towards the diversity when looking for a “sweat threat” behavior in the area of sweet desert toppings, is much more standardized. Next marketing strategy would be evaluating a different distribution channel, like gastronomy and hotel business, catering companies, restaurants and confectioneries.

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EFFECT OF GENDER, AGE AND OCCUPATION OF JUDGING CONSUMERS ON SENSORY EVALUATION OF VARIOUS LAMB MEAT FOODS

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ABSTRACT: In a research aiming to improve lamb meat quality and quantity of 8 different genotypes were examined. Various lamb meat foods were developed based on genotypes to study the differences among them: Wiener sausage (8 kinds); marinated, smoked and cooked ham (8 kinds); kitchen ready, seasoned, marinated, sliced and roasted lamb meat (8 kinds); whole roasted (restaurant style) lamb leg (4 kinds).
Altogether 285 (60% male and 40% female) randomly selected heads of consumers were included in sensory evaluation/ judging of the products. The consumers represented different age groups: below 20 years; between 21-30; 31-40; 41-50; 51-60; 61-70, and above 70 years. The groups of consumers were involved in: sheep farming; other animals' farming; other agriculture; industry; catering, education, student, other service; administrative department of the state, and others.
The stringiness, flavour and smell were judging in food sensory evaluation. In stringiness and flavour five (from 1 to 5), and concerning smell three (from 1 to 3) categories were created according to decreasing quality.
Analyses of variance were calculated to estimate the effects of lamb genotype; the gender, age, and occupation of judging consumers on sensory evaluation of the various meat foods.
In conclusions, it was stated that new lamb meat foods were highly appreciated by consumers. Consumers made definite distinctions among genotypes according to flavour, stinginess and smell. There were significant interactions between gender, age and occupations of consumers as well as sensory evaluation of various lamb meat foods.
Key words: *lamb genotypes, meat products, consumer, sensory evaluation*

INTRODUCTION

The various authors stated that the most dominant effects modifying the sensory values of the lamb meat are the genetics (Fahmi et al, 1992; Horcada et al, 1998; Ducket and Kuber 2001; Hofmann et al, 2003; Brzostowski et al, 2004; Teixeira et al 2005; Osman and Aldosari 2006), the feeding regime (Melton ,1990; Fischer et al, 2000; Sanudo et al, 2000; Corraiz et al, 2000; Bunch et al, 2004; Revilla et al, 2009), the slaughter weight (Horcada et al, 1998; Berian et al, 2000), and the gender of lambs (Teixeira et al, 2005; Lind et al, 2011).
Consumers could distinguish lambs according to their origin and their preferences. Dominantly well trained panellists were making sensory examinations of various kinds of meat (including lamb meat), but even on family level the untrained panellist could make differences among breeds of lamb according to their preferences. The cultural background and previous experiences or knowledge affect the preferences of the panellist (Ward et al., 1995). The flavour ratings appear to be largely related to the panellist's preference and previous exposure to lamb (Sanudo et al., 1998, 2000).
In many studies stated that the gender of the lamb has significant effect on meat quality, but the acceptance of the lamb meat according to gender of the panellist has not been studied. Also limited information is available about the effect of age and the profession of the panellist on the ratings of sensory attributes of lamb meat.
In Hungary, lamb meat and mutton are dominantly consumed as stew, or rarely a kind of soup, but other kinds of foods are not really made. The main aim of this present study was to examine the acceptance of various kinds of foods made from lamb meat by the not well

trained panellists. The other aim of this study was to investigate the ability of panellist to distinguish breeds; as well as the effect of gender, age and profession of panellists on the sensory evaluation.

MATERIALS AND METHODS

The animals

In a part of experiment serial, lambs belonging to different genotypes were indoors fattened ad libitum on pelleted feed with limited grass hay supplement: Transylvanian Racka ewes were harem mated by rams of following terminal breeds: Transylvanian Racka (1), British Milksheep (2), Charollaise (3), Ile de France (4), German Blackhead Mutton Sheep (5), German Mutton Merino (6), Suffolk (7) and Texel (8). Forty lambs (20 males + 20 females) per genotypes were fattened up to 27-30 kg of live weight category, on approximately 60 days after weaning at 16-18 kg body weight. Half of the lambs (10-10 male and female) from each group were slaughtered.

The preparation of samples

The carcasses were classified using S/EUROP grading system and kept 24 hours after slaughtering at +4 ^{o}C temperature. The carcasses were halved and the right halves were dissected, boned and measured. Meat were handled by genotypes, samples for different products were separately packed into plastic bags, and after slow cooling they were frozen and kept at -20 ^{o}C temperature until preparation.

The meat was gradually de-frosted and three products were developed and manufactured at Biharnagybajom Slaughterhouse and Meat Processing Ltd: Wiener sausage (8 kinds); marinated, smoked and cooked ham (8 kinds); kitchen ready seasoned, marinated, sliced and roasted lamb meat (8 kinds). The manufacturing process was the same in all genotype.

The prepared meat products were aged for three days, four weeks and two days, respectively in the previous order. The hams were smoked for 24 hours following four weeks of aging, and after two days of rest they were cooked, than cooled down and kept at 8 ^{o}C until presentation.

The developed products were promoted and tasted at Farmer Expo Exhibition (Debrecen). The Wiener sausages were boiled only before sensory study and kept served at 40 ^{o}C. The seasoned and sliced lamb meat were roasted on 170 ^{o}C right before sensory test and offered at 40 ^{o}C to the participants of the sensory panel. The lamb hams were warmed up naturally and served at 18 ^{o}C.

For the preparation of the whole roasted lamb leg (restaurant style) two heads of purebred Transylvanian Racka, British Milksheep F_1, Charollaise F_1, and Texel F_1 were slaughtered two days prior presentation and sensory study, and prepared in the oldest and most famous restaurant in Debrecen (Arany Bika Restaurant). The legs were kept at 60 ^{o}C until offer to sensory evaluation.

Samples (3 x 1.5 x 2.0 cm cubes) were made from various products and put on a preheated plate. These samples were coded with a randomized three-digit number.

The sensory panel and examination

The sensory panels were created from visitors of the abovementioned exhibition. The first three products were sensory examined every days around midday by 80 (44 male + 36 female – Wiener sausage); 82 (50 male + 32 female - marinated, smoked and cooked ham); and 64 (37 male + 27 female – marinated, seasoned, sliced and roasted lamb meat) persons. The fourth product was qualified one hour before the official reception of exhibition by 39 (27 male + 12 female – whole roasted lamb leg) panellists.

The members of panels were educated about quality examination of various meat products. Three traits were requested to be rated as follows: ***stringiness*** /1 very soft; 2 soft; 3 nether soft nor stringy; 4 stringy; 5 very stringy/; ***flavour*** /1 very delicious; 2 delicious; 3 satisfying; 4 not satisfying; 5 foul flavour/; and ***smell*** /1 perfect; 2 sufficient; 3 insufficient/.

The panellists individually filled in a form concerning kinds and number of products, including the following information about panellists: gender (male, female); age (under year 20; between 21-30; 31-40; 41-50, 51-60; 61-70, above 70); as well as profession (sheep farmer/shepherd; involve in other kind of animals' farming; other agriculture; industry, catering; education; student, other service; administrative department of the state, and others).
Analyses of variance were calculated to estimate the effects of lamb genotype, age, occupation and gender of judging consumers on sensory evaluation of various meat foods.

RESULTS AND DISCUSSION

The three developed new lamb meat products and the whole roasted lamb leg made in restaurant style were evaluated separately (Table 1-3).

Table 1. Sensory evaluation by gender of judging consumers and by breeds (average of scores)

Food		1	2	3	4	5	6	7	8
Wiener sausage									
Stringiness -	male	2.56B	2.55aB	2.23C	2.59B	2.57A	2.66A	2.50B	2.30C
	female	2.58B	2.36C	2.47aC	2.61B	2.72aA	2.75aA	2.56B	2.56Ba
Flavour -	male	2.09C	2.30aA	2.14B	2.09aC	2.18aB	2.16aB	2.02C	2.09aC
	female	2.11A	1.86C	2.14A	1.94B	1.97B	1.89C	2.11A	1.83C
Smell -	male	1.59B	1.66aB	1.73aA	1.66aB	1.64B	1.66B	1.66aB	1.68aB
	female	1.53C	1.50C	1.58B	1.56B	1.64A	1.67A	1.56B	1.47C
Ham									
Stringiness -	male	2.32B	2.24aC	2.38A	2.31aC	2.26aC	2.34B	2.40aA	2.14C
	female	2.34B	2.13C	2.50aA	2.06C	1.91D	2.25C	2.09C	2.16C
Flavour -	male	2.14A	2.08A	1.92B	2.04A	1.94aB	2.14aA	2.12A	1.92aB
	female	2.38aA	2.13B	2.19aB	2.06B	1.69C	1.84C	2.00B	1.72C
Smell -	male	1.66aB	1.62aB	1.62B	1.51aC	1.46aC	1.64aB	1.72aA	1.58aB
	female	1.56B	1.50B	1.69A	1.34C	1.31C	1.50B	1.59B	1.34C
Roasted meat									
Stringiness -	male	2.38B	2.38B	2.24C	2.03D	2.38B	2.54A	2.35B	2.32B
	female	2.37B	2.39B	2.04C	2.22C	2.78A	2.41B	2.37B	2.44B
Flavour -	male	1.86B	1.97A	1.81B	1.76C	2.00A	1.76C	1.89B	1.84B
	female	1.85C	2.04B	1.85C	2.04B	2.19A	1.96C	2.04B	1.89C
Smell -	male	1.41C	1.49B	1.46B	1.38C	1.41C	1.59A	1.54A	1.46B
	female	1.59C	1.67B	1.56C	1.70B	1.89A	1.63B	1.52C	1.41C
Roasted leg									
Stringiness -	male	2.07C	2.11C	2.78A	-	-	-	-	2.22B
	female	2.00C	2.08C	3.17aA	-	-	-	-	2.83aB
Flavour -	male	2.11aC	2.48A	2.00C	-	-	-	-	2.22B
	female	1.92D	2.75aA	2.50aB	-	-	-	-	2.42aC
Smell -	male	1.08C	2.07aA	1.81B	-	-	-	-	1.84B
	female	1.25aC	1.75B	1.83B	-	-	-	-	2.00aA

Different superscript means significant differences (P<0.05) between genders (a) between breeds (A,B,C,D)

Wiener sausage

The gender, age and profession of panellist had definite effects on judgements concerning stringiness, flavour and smell of various kinds of sausages. Reasonable deviations were observed among studied genotypes. In the case of stringiness the British Milksheep F_1, in flavour the Ile de France F_1, and in smell the purebred Transylvanian Racka reached the best result.
Bigger differences were observed concerning stringiness and flavour among studied genotypes, while smaller deviations found in smell according to gender of panellists. On the contrary, significant difference was only received in smell between judgements of male and

female panellists. These data were modified by breeds (Teixeira et al 2005; Osman and Aldosari 2006).
It seemed that the age of panellists essentially modified values of scores regarding stringiness and flavour. The judgements of older panellists fundamentally differed from scores given by younger panel members in smell, as for the latter ones the lamb meat was dominantly unfamiliar.
The profession definitely modified judgements. In flavour there was strong difference between scores given by shepherds and other professions. However, concerning smell judgements of panellists working in industry, education, and others services were significantly different from other members of panel.

Table 2. Sensory evaluation by occupation judging consumers (average of scores)

Food	1	2	3	4	5	6	7	8
Wiener sausage								
stringiness	2.71a	2.25b	2.56a	2.65a	2.56ab	2.83a	2.96ac	2.30b
flavour	2.36a	1.82b	2.24ac	2.13a	1.81b	1.98bc	2.02bc	2.05b
smell	1.79a	1.61ab	1.70ac	1.42b	1.50ab	1.45b	1.54bc	1.61bc
Ham								
stringiness	2.59a	2.15bc	2.35ab	2.06bc	2.42abc	2.13bc	2.00c	2.11c
flavour	2.36a	2.00b	2.07b	2.00b	2.13ab	1.84b	1.94b	1.90b
smell	2.02a	1.49bd	1.67c	1.50bcd	1.67bc	1.38bd	1.41bd	1.34d
Roasted meat								
stringiness	2.79a	2.31b	2.38a	2.25ac	2.50bc	2.36ac	2.38ac	2.23bc
flavour	2.21a	1.79b	2.05ac	2.13ac	1.75ac	2.09a	2.08ac	1.82c
smell	1.79a	1.43bc	1.90a	1.67ac	1.25b	1.62a	1.43b	1.52bc
Roasted leg								
stringiness	2.42a	2.41a	2.00ab	-	1.38b	2.63a	2.50a	2.35a
flavour	2.52a	2.09b	2.00abc	-	1.13c	3.00a	2.30ad	2.15ad
smell	1.96a	1.68b	1.25b	-	1.50ab	1.88ab	1.60b	1.80ab

Different superscript in a row means significant differences (P<0.05)

Marinated, smoked and cooked lamb ham

There were significant differences among studied genotypes in stringiness, flavour and smell. In all three traits differences were observed between opinions of male and female panellists, however, these differences were only significant in smell, but in the case of comparing breeds significant differences could be observed (Teixeira et al 2005; Osman and Aldosari 2006).
The age had strong effects on scores in stringiness and flavour. The judgements of age category 21-30 and above 70 years definitely deviated from scores given by others. In smell differences were observed among age groups, and score values were higher in older member of panel.
There were significant differences between shepherds and other professional groups. This tendency could also be observed in smell, however, judgements of other agriculture group were closer to shepherds.

Kitchen ready, seasoned, marinated, sliced roasted lamb meat

Bigger differences were observed among genotypes in stringiness and flavour, while concerning smell smaller deviations were found. The gender concerning summarised values had strong effect on stringiness and flavour, and in smell the differences could also be observed. At the same time, these differences were only significant in smell. Apart from them, there were significant breed differences among the scores (Horcada et al, 1998; Ducket and Kuber 2001; Hofmann et al, 2003; Brzostowski et al, 2004).
Studying age panelists below 30 years gave different judgements than other age groups concerning stringiness and flavour. Apart from these, effects of age groups could also be followed in smell scores.

The profession of panelists resulted less determining effects on stringiness and flavour as it was experienced concerning Wiener sausages. On the contrary, there were big differences among professional groups concerning smell.

Whole roasted lamb leg (restaurant style)

Differences among genotypes could also be followed in this product concerning all three quality traits. The gender definitely modified score values of stringiness and flavour, and differences were also found in smell in the cases of summarised values. However, because of high standard deviations gender effect were not significant. Apart from them there were significant differences among breeds (Teixeira et al 2005; Osman and Aldosari 2006).
Comparing age groups no significant differences were found in stringiness. On the contrary, in smell definite age group differences were observed. Concerning smell scores groups above 40 years were significantly different from those ones originated from other age groups. In stringiness and flavour judgement of catering group (like cooks) was significantly different from other profession groups. Concerning smell scores of shepherds significantly deviated from other profession groups.

Table 3. Sensory evaluation by age of judging consumers (average of scores)

Food	1	2	3	4	5	6	7
Wiener sausage							
stringiness	2.09a	2.46bd	2.49bd	2.80c	2.54d	2.57bcd	2.38abd
flavour	1.75a	2.26bc	2.01ac	2.25bc	1.94ac	2.03ac	2.22c
smell	1.75a	1.66a	1.61a	1.63a	1.50b	1.73ac	1.34c
Ham							
stringiness	2.38abc	2.61a	2.28bc	2.39ab	2.15c	2.13c	1.70d
flavour	2.08ab	2.23a	2.13ab	1.94b	2.02ab	1.98b	1.58c
smell	1.67ac	1.78a	1.40bd	1.49cd	1.42d	1.80a	1.33d
Roasted meat							
stringiness	2.15a	2.72b	2.18a	2.43a	2.19a	2.39a	2.25a
flavour	1.46a	2.20b	1.84cd	2.00bd	1.79cd	2.13b	1.63ad
smell	1.15a	1.73b	1.36c	1.69d	1.40c	1.89b	1.38ac
Roasted leg							
stringiness	-	2.30a	2.13a	2.56a	2.45a	2.19a	-
flavour	-	2.60a	1.75b	2.75a	2.20ab	1.94b	-
smell	-	1.80a	1.75a	2.06ab	1.70ac	1.59ac	-

Different superscript in a row means significant differences (P<0.05)

CONCLUSIONS

Summarising the results of examinations we could draw the following conclusions down. The general acceptance of the various lamb meat foods was much higher than the expectations; the acceptance of the lamb meat could be improved by introducing new kinds of foods. Strong differences could be followed in the evaluations of stringiness, flavour and smell according to the gender of the panelists. The age and the profession of the panellists have significant effects on the score given to various foods, as their preference and knowledge about various kinds of meats were different. There were significant differences among the panellists in evaluation of stringiness, flavour and smell values. The panellists were able to distinguish the various breeds in the evaluation of foods.

ACKNOWLEDGEMENTS

This paper is a part of the results within the project GVOP-3.3.1.-2004 "*Bio-juhhús termelés fejlesztése"* (Development of bio lamb meat production), financed by the National Research and Technology Office, Hungary.

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FUNCTIONAL AND TRADITIONAL FOOD: THE FIRST RESULTS BASED ON A STUDY OF CONSUMERS PROFILES IN THE WESTERN BALKANS

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ABSTRACT: The research is based on the consumer quantitative survey performed on general population 18+ in six Western Balkan Countries (WBC - Bosnia and Herzegovina, Croatia, Macedonia, Montenegro, Serbia and Slovenia) at the end of September – beginning of October 2010. The instrument used in this survey was a structured questionnaire. The stratified three-staged random representative sample was applied (N=3085). Based on reported frequency heavy traditional food consumers - who simultaneously reported lower level of functional food consumption (N_t=723), and heavy functional food consumers - with lower level of traditional food consumption (N_f=400), were identified. Descriptive analysis (parametric and non-parametric) was conducted with the aim to investigate statistically significant differences between two profiles of identified consumers' groups in WBC. The study addresses the opposite consumers' profiles. The recommendations for food marketers are emphasized in the concluding remarks.
Key words: *traditional food, functional food, motives, socio-demographics, consumer profile, WBC.*

INTRODUCTION

The concept of traditional food is multidimensional. Seen from different points of view and recognized in different forms, this concept links tradition with food consumption. Traditional food is usually defined as a typical food of local origin, home-made or on-farm produced (Ricketts et al., 2006; Vogt and Kaiser, 2008; Wilson and Fearne, 2000; Hamermesh, 2007). Additionally, traditional products constitute an important element of European culture, identity and heritage, which has been recognised both at scientific and political levels (Committee of the region 2010, Communication of the European Parliament 2011). At the same time, the urban diet changes the occasions and the frequency of consumption of the traditional food. Additionally traditional food is reputed as not so healthy in some regions (Ambelard et al, 2010).

On the other hand, functional food gains in importance recently. Marketed as novel, this food type has been often seen as an opposite to the traditional food. Produced by leading multinational companies, functional food is considered as a much more global concept. Simultaneously, there is an increasing importance of health aspects in food consumption (Diplock et al, 1999; Sijtsema et al, 2003, De Jong et al, 2003; Niva & Mäkelä 2005). This paper aims to investigate differences regarding motives toward food choice and main socio-economic and demographic characteristics between two groups of consumers in Western Balkan Countries (WBC) – heavy traditional and heavy functional group. The food choice motives were analyzed by Milosevic and others (Milosevic et al, 2012). However, profiling of chosen consumer groups can improve understanding of contemporary food consumption and possible dynamics which are of crucial importance for policy makers.

THE SAMPLING PROCEDURE, RESEARCH INSTRUMENT AND METHODS

Consumer quantitative survey was performed on general population 18+ in WBC (Bosnia and Herzegovina, Croatia, Macedonia, Montenegro, Serbia and Slovenia). The sampling was based on the data from Census and estimated population dynamics. The stratified three-staged random representative sample was applied. The primary sampling units (PSU) were polling station territory, the secondary sampling units (SSU) households and the tertiary sampling units (TSU) respondents – the adult member per each chosen household. The Troldahl and Carter (1964) modification (T-C) of the Kish technique was used for data collection. The total sample was 3085, whereas the valid sample for our study was 1123 respondents who stated high frequency of traditional or functional food consumption.
The instrument used in this survey was a structured questionnaire consisting of several separate sections, including motivation toward food in general (Food Choice Questionnaire - FCQ), some specific questions about traditional and functional food and general consumer's socio-economic and demographic characteristics. The motivation was measured by 36 items divided into nine groups (*health, mood, weight control, natural content, convenience, sensory appeal, price, familiarity and ethical concerns*) on a five points' Likert scale. As the concept of functional food is a new on the emerging WBC market, the respondents were previously advised on what was typically assumed under this term. Also, several examples were presented before respondents were asked to complete the survey. Consumers' socio-economic and demographic characteristics relevant for this study includes their gender and age, level of education, number of household members, type of settlement (rural/urban), employment, height and weight - body mass index (BMI), as well as their self-assessed economic and health statuses. The dependent variable was self-reported frequency of consumption of the studied food categories.
In order to find out differences between heavy traditional and functional consumers in WBC, the final sample was reduced: consumers who simultaneously stated high consumption of both foods were eliminated. Consequently, the relevant sample consists of consumers who stated higher consumption of one food group than other – e.g. the *heavy traditional* profile represent consumers who reported higher traditional food consumption and simultaneously lower functional food consumption (Nt=723), and the *heavy functional* group *visa versa* (Nf=400). A descriptive statistical analysis is conducted aiming at investigating all statistically significant differences between both consumers' profiles. The differences between the two groups related to a number of socio-demographic and attitudinal variables were measured by parametric (One-Way ANOVA) or non-parametric (Chi Square) statistical tests.

RESULTS AND DISCUSSION

Heavy traditional are more represented than *heavy functional foo*d consumers in our sample (Table 1). Almost 2/3 of all respondents belong to the first, while 400 respondents were found in the second group. Generally, when compared with the *heavy functional*, the *heavy traditional food* consumer is more frequently man than women, slightly older on average (44 and 48 years old respectively), significantly more accessible to agricultural resources, secodnary or less educated in general and more exposed to problem of overweight, even obesity (percent of people with BMI=>32 is recorded at 8.44% in this group). The *heavy functional food* consumer profile is obviously completely different, like a picture in a mirror. However, this analysis supports our study with higly rough description of two consumers' profiles in WBC. Additionally, the consumers' profiles might differ on the national level which was not in the focus of this paper analysis.
Further statistical analysis indicates the opposite characteristics of two 'heavy consumers' groups in WBC. Differences were found at the highest level in almost all attributes (Table 2). Obviously two consumer groups are significantly different regarding gender, age, type of household and settlement, level of education and sophisticated characteristics such as self-

perceived economic status, health status (measured by self-reported current state and BMI) as well as importance of social influences (rated by the immediate environment interactions).

Table 1. The sample structure

The sample characteristic	Heavy traditional food consumers		Heavy functional food consumers	
	Total	%	Total	%
No of respondents	723	64.38	400	35.62
Gender				
Male	317	43.85	138	34.5
Female	406	56.15	262	65.5
Age				
<30	136	18.81	103	25.75
30-60	393	54.37	215	53.75
60>	194	26.82	82	20.05
Settlement				
Urban	405	56.02	285	71.25
Rural	318	43.98	115	28.75
Agricultural households	167	82.67	35	17.33
Employment*				
Employed	278	38.45	179	44.75
Unemployed	445	61.55	221	55.25
BMI**				
<25	325	45.20	218	55.90
25>	394	54.80	172	44.10
Education				
Secondary or less	600	82.99	284	71.00
Higher	123	17.01	116	29.00
Country				
Bosnia and Herzegovina	135	65.22	72	34.78
Croatia	107	65.64	56	34.36
Macedonia	174	77.68	50	22.32
Montenegro	63	43.45	82	56.55
Serbia	184	76.35	57	23.65
Slovenia	41	28.67	102	71.33

**Unemployed group includes pensioners*
***Calculated based on self-reported weight and height*

Differences in employment were found at 5% significant level. Namely, *the heavy traditional food* consumers are more often unemployed than the *heavy functional food* consumers in WBC. It is also important to emphasize that unemployment in our case also signifies that people are retired which is again linked to their age. Additionally, pensioners are slightly more represented in the sample of the *heavy traditional* than *functional food* consumer group in WBC (28.91% and 25.25% respectively). However, it is equally important to emphasize the structure of unemployed *heavy functional food* group. It comprises more of pupils and students (10.75%) compared with the structure of *heavy traditional food* consumer group (6.64%). It might indicate a food consumption pattern dynamic in WBC.
The rank of motivations toward food consumption was almost the same for both consumers groups in WBC. After ‘health’ - ‘mood’, ‘sensory appeal’, ‘convenience’, ‘natural content’ and ‘price’ were most important. The least important factor was ‘ethical concerns’ for both groups. Slight difference was noticed when importance of ‘weight control’ and ‘familiarity’ had been considered as the motives for food choice.

Table 2. Differences in consumers` profile in the Western Balkans, 2010

Variables	Definition	Heavy traditional food consumers	Heavy functional food consumers	Statistical tests
		Mean (standard deviation)		One-way ANOVA
Age	In years	48.30 (17.275)	44.42 (17.880)	F=12.677***
Economic status	5 point scale	3.08 (0.853)	3.33 (0.804)	F=23.029***
Health status	5 point scale	3.59 (0.962)	3.78 (0.888)	F=9.932***
BMI	self-reported	25.73 (4.123)	24.68 (4.091)	F=16.574***
Social influences /functional food	5 point scale	0.22 (0.974)	0.70 (0.938)	F=63.244***
Social influences /traditional food	5 point scale	1.28 (0.810)	0.81 (0.864)	F=82.073***
		Frequencies (share)		Chi-Square
Gender	0 = male	317 (43.85%)	138 (34.50%)	9.332***
	1 = female	406 (56.15%)	262 (65.50%)	
Education	0 = secondary or less	600 (82.99%)	284 (71.00%)	22.090***
	1 = college/university	123 (17.01%)	116 (29.00%)	
Type of settlement	0 = urban	405 (56.02%)	285(71.25%)	22.225***
	1 = rural	318 (43.98%)	115 (28.75%)	
Type of household	0 = non-agricultural	556 (77.32%)	365 (91.25%)	36.174***
	1 = agricultural	167 (22.68%)	35 (8.75%)	
Employment	0 = unemployed	445 (61.55%)	221 (55.25%)	4.234***
	1 = employed	278 (28.45%)	179 (44.75%)	

Significant at 10% significant level. ** Significant at 5% significant level. * Significant at 1% significant level*

However, in order to find out if the significant differences exist in motivation toward traditional and functional food consumption, one-way ANOVA test was applied. The results are presented in Table 3. Differences between two consumers groups were found to be significant at 1% level in 'health' motivation, and 'convenience'. Functional food consumers were more oriented toward 'health' motive and 'weight control' while choosing food than traditional consumers in WBC. Simultaneously, they do care more about 'convenience' in food consumption. Contrary to these findings, both consumers groups insist equally on hedonic aspects - 'mood' and 'sensory appeal' while making food choices. Moreover, heavy traditional food consumers express less favoured attitudes toward 'ethical concerns' – food origin, environmental protection and small-scale production. Regarding this motive, statistically significant difference was found at 5% level. Finally, the heavy traditional consumer group insisted more on 'familiarity', while heavy functional consumer group was more oriented toward naturalness. However, the finding might be controversial: the local, on-farm production of traditional food usually relays on strong consumer ethical orientation, while functional food is more exposed to new technologies which might not insist on pure naturalness.

Table 3. Differences in motivations toward food consumption in WBC, 2010

Motivation	Heavy traditional food	Heavy functional food	Test
	Mean (standard deviation)		One-way ANOVA
Factor 1—Health	8.56 (3.656)	9.75 (3.335)	F=28.826***
Factor 2—Mood	5.41 (4.367)	5.51 (4.963)	F=0.108
Factor 3—Sensory Appeal	5.33 (2.345)	5.42 (2.313)	F=0.402
Factor 4—Convenience	4.48 (3.785)	5.13 (3.573)	F=7.681***
Factor 5—Natural Content	3.60 (2.212)	3.85 (2.115)	F=3.385*
Factor 6—Price	3.44 (2.255)	3.48 (2,264)	F=0.088
Factor 7—Familiarity	2.68 (2.337)	2.41 (2,553)	F=3.195*
Factor 8—Weight Control	1.91 (2.726)	2.60 (2.709)	F=16.559***
Factor 9—Ethical Concern	0.47 (2.992)	0.94 (2.982)	F=6.184**

Significant at 10% significant level. ** Significant at 5% significant level. * Significant at 1% significant level*

CONCLUDING REMARKS

Our research addresses the most important marketing issues of creation of competitive and safe food products by introduction of new technologies in traditional food production in the Western Balkans. The findings are based on comparison of heavy consumer profiles – traditional *versus* functional food (the later represents the approximation of novel food in the region). The analysis is based on identification of heavy consumers, important in profiling of marketing diffusion strategies.

Differences between two consumer profiles were confirmed to be significant in each socio-demographic and economic attribute applied in our research. Simply, two consumer groups seem to be completely opposite: (1) younger, educated, urban, female and more employed are the main socio-economic characteristics of WBC *heavy functional food* consumer group; (2) older on average, secondary or less educated, rural, more often mail, members of agricultural households and unemployed represent *heavy traditional food* consumer profile. Although so different, two consumer groups almost identically rank the motivations toward food choice. While choosing food, both groups in WBC were motivated by health, mood and sensory appeal, followed by convenience, natural content and price. No matter whether they choose traditional or functional food, consumers are simply convinced about healthiness and tastefulness of their choice, including hedonic aspects. Additionally, while insisting on product naturalness, both groups consider the specific food convenient (easy to prepare/buy/consume).

However, the important differences between consumers were found in health motivation, weight control and convenience (at 1% significant level), as well as due to ethical concerns (significance at 5%), familiarity and natural content (at 10% significant level). *Heavy functional food* consumers are more oriented toward health, weight control and convenience. They do more care about naturalness and ethical concerns in food production than *heavy traditional food* consumers in the region. Food marketers should take care about the 'natural content' of food as consumers in the region do not want to leverage between "artificial" foods ingredients and other motives while consuming food. On the other hand, lower ethical concerns of heavy traditional food consumers might indicate lower potentials for promotion of regional food concept. The traditional food might be used as a term for low marketed goods home-produced by traditional recipes. However, the meaning of traditional food for consumers in different countries of the Western Balkans is highly debatable (Stojanovic & Barjolle, 2012). The further research should also contribute explanation of term "traditional food" in the region.

ACKNOWLEDGEMENTS

The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7 2007-2013) under grant agreement n°212 579 – FOCUS-Balkans Project and the Republic of Serbia Ministry of Science under Project no. III 46001 (2011-2014).

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SENSORY PROPERTIES OF CIDER, DEPENDING ON THE ADDED YEAST STRAINS

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ABSTRACT: Sensory properties of fermented beverages are an important quality indicator. The taste and aroma of the products are formed not only from the raw material, but also important is the added yeast, since it affects on the sensory properties of the finished product. The aim of current research is to evaluate the influence of different yeast strains on the sensory properties of cider. Apple variety 'Lietuvas Pepins' juice was fermented with four different commercial yeast strains *Saccharomyces bayanis* yeasts 'EC-1118'and 'Cider yeast', *Saccharomyces cerevisiae* yeasts '71B-1122' and 'K1V-1116'. The sensory evaluation of samples was carried out by experts and intensity of sensory properties (namely, clarity, apple, fruit and yeast aroma, apple, yeast, sour, astringent and bitter taste) using line scale was evaluated. The data obtained in present study shows that yeast strains has a significant influence to the sensory properties of cider. Higher intensity of apple and fruit taste and aroma characteristics showed samples fermented with '71B-1122' and 'K1V-1116' yeasts, whereas samples fermented with 'EC-1118'and 'K1V-1116' showed higher intensity of yeasts aroma and taste. Generally better results showed sample fermented with '71B-1122' yeast. Results of current research approve that choice of the yeast strains are very important for developing technology of the cider.
Key words: *cider, yeast strains, sensory properties*

INTRODUCTION

Apple production is one of the largest fruit-growing sectors of Latvia. Most apples are sold fresh, but part of the apple, which does not meet the requirements of the market, has to be processed and therefore, fermented apples beverage production could be good perspective. The quality of fermented drinks like cider is a characteristic originated from the presence of aroma compounds in a product (Mangas et al., 1996), that are influenced by several factors, namely apple variety, yeast strains, fermentation conditions, the production process and fining treatments (Hidalgo et al., 2004; Martınez-Rodrıguez and Polo, 2003). The fermentation of apple must is a complex microbial reaction involving the sequential development of various strains of yeasts and bacteria (Duenas et al., 1994). Among these micro-organisms, yeasts are primarily responsible for alcoholic fermentation. The different yeast species developed during fermentation and their dynamics and frequency of appearance determine the taste and flavour characteristics of products (Cabranes et al., 1997). Ethanol and glycerol are quantitatively the dominating alcohols, followed by higher alcohols and esters. The main ester produced during alcoholic fermentation is ethyl acetate, but other esters of fusel alcohols and medium chain fatty acids also appear. Two main species, *Saccharomyces cerevisiae* and *Saccharomyces bayanus*, are currently recognized among wine yeasts (Masneuf-Pomarède et al., 2007; Naumov et al., 2000). Different strains of *Saccharomyces cerevisiae* can produce significantly different flavour profiles when fermenting the same must. This is a consequence of both, the differential ability of wine yeast strains in releasing various volatile compounds from grape precursors, as well as the differential capacity to synthesize new yeast-derived volatile compounds (Swiegers et al., 2006; Ugliano et al., 2006). Also in our previous investigations significant influence of the yeast strain on the final chemical and volatile composition was observed (Riekstina-Dolge et al., 2011). Formation of a new product should be designed with high-quality beverage taste of the consumer sensory evaluation. In order to match the instrumental analysis with the consumer requirements, a sensorial profile of the cider is necessary. Sensory quality is

related to consumer acceptance and confidence in the product, being defined by the interaction between the food and the consumer. Thus, sensory quality depends on both the sensory characteristics of the food and how consumers perceive them (Cardello, 1995; Costell, 2002; Ares et al., 2009). Sensory descriptive analysis is a primary tool of food scientists, which involves the evaluation of both the qualitative and quantitative sensory characteristics of products (Meilgard et al., 1999).
The aim of current research is to evaluate the influence of different yeast strains on the sensory properties of cider.

MATERIAL AND METHODS

Raw materials

In these experiments apples of variety 'Lietuvas Pepins' grown in the Latvia State Institute of Fruit and harvested in October 2010 were used. Juice was obtained by mechanical press *Voran Basket Press 60K* (Voran Maschinen GmbH, Austria). For stabilization of juice before fermentation 'Tannisol' (Enartis, Italy) was added. Tannisol capsules consist of potassium metabisulphite (added amount to juice – 9.5 g 100 L^{-1}), ascorbic acid (0.3 g 100 L^{-1}) and tannin (0.2 g 100 L^{-1}). Sulphites have various permitted uses, their primary function is as a preservative and antioxidant to prevent or reduce spoilage (Fazio and Warner, 1990) and they help to stabilize product colour and inhibit discolouration, thereby improving the appearance and flavour of many foods during preparation, storage and distribution (Adams, 1997).

Fermentation conditions

Fermentation was performed using four commercial yeasts – *Saccharomyces bayanis* yeast EC-1118 (Lalvin, Canada), *Saccharomyces bayanis* 'Cider yeast' (Youngs Home Brew., UK), *Saccharomyces cerevisiae* yeast 71B-1122 (Lalvin, Canada) and *Saccharomyces cerevisiae* yeast K1V-1116 (Lalvin, Canada). Fermentation was carried out at 16±1 °C for 28 days. The apple juice was fermented in a glass bottles (n=5) with a volume 750 ml in the laboratories of Latvia University of Agriculture, Faculty of Food Technology. For analysis purpose the average juice samples were combined from 5 bottles in equal proportions. All of ciders were performed immediately after 28 day fermentation. Chemical parameters of cider are presented in Table 1.

Table 1. Chemical parameters of ciders

Yeast variety	Abbreviation	Titratable acidity, g L^{-1}	Soluble solids, g L^{-1}	Alcohol, % vol.
EC-1118	SB1	9.40±0.04	7.70±0.02	5.4±0.05
Cider yeast	SB2	8.40±0.08	6.40±0.03	5.6±0.06
71B-1122	SC3	8.02±0.06	5.14±0.01	5.9±0.03
K1V-1116	SC4	8.32±0.08	15.40±0.02	4.3±0.08

Sensory analysis

Sensory evaluation of cider was carried out with experts (nine women and two men, aged 21 to 51 years). Experts are specialists in the field of beverage technology and experienced in sensory analysis of beverages.
Two methods of sensory analysis were used:
1) identification of cider flavours using characteristic descriptors divided in 12 classes (Table 2) developed by Willams (1975);
2) line scale method for measuring intensity of sensory attributes.

Table 2. Characterization of cider flavours (Williams, 1975)

Class	General characterization	Characterization of subclasses
1	Sour, acidic	Acidic, apple (sharp) acid, vinegar, lactic (soft) acid, citrus sour
2	Aromatic, fragrant, fruity, floral	Alcoholic (fusel), solvent-like (plastics, can-liner, acetone), estery (pear drops, apple-like with aniseed note, light fruity), fruity (citrus fruit, banana, blackcurrant, melon, pear, forest fruit, culinary apple, bittersweet apple), acetaldehyde, floral (rose-like, perfume-like, geranium
3	Spicy, nutty, grassy	Spicy (resinous, woody, spicy bittersweet), nutty (walnut, almond), grassy
4	Caramelised, toasted	Caramel (molasses, raisins), burnt (toasted, rubbery)
5	Chemical	Phenolic (tarry, carbolic, antiseptic, iodoform), plastic, oily (mineral oil, vegetable oil), indole
6	Soapy, fatty, diacetyl, rancid	Fatty acid (soapy, cheesy, rancid butter), butterscotch, rancid
7	Sulphury	Sulphury, sulphur dioxide, sulphidic (rotten egg, drains, autolysed, burnt rubber, shrimp-like, cooked vegetable, cooked cabbage), yeasty
8	Oxidised, stale, musty	Stale, catty, papery, leathery, sherry-like, mouldy (earthy, musty), biscuit
9	Sweet	Honey, artificial (saccharin), vanilla, syrup
10	Bitter	Bitter
11	Mouthfeel	Alkaline, metallic, astringent (drying), powdery, creamy, carbonation (flat, gassy), warming
12	Fullness	Body (watery, characterless, satiating, thick)

Experts also evaluated all samples using 12 cm line scale to rate the intensity of nine descriptors: clarity, apple aroma, fruit aroma, yeast aroma, apple taste, sour taste, yeast taste, astringent and bitter taste. Scale of clarity was defined as clear (on left side of line) and cloudy (on right side of line). For other parameters left side was described as 'attribute not perceptible' and the right side 'attribute strongly perceptible'.
Analysis of variance was performed by ANOVA procedure and $p<0.05$ was considered as statistically significant and principal component analysis was performed with the software SPSS 17.00 for Windows.

RESULTS AND DISCUSSION

Identification of cider flavors

In analyzed samples experts identified descriptors of eight classes of cider flavours: 1) sour, acidic, 2) aromatic, fragrant, fruity, floral, 3) soapy, fatty, 4) sulfury, 5) oxidased, stale 6) bitter, 7) mouthfeel, 8) fullness (Table 3).
In all analysed samples experts identified following flavours: vinegar acidity, citrus sour, apple like esters, astringency and satiating body. Only for two descriptors – citrus fruit flavour and bitterness, differences between *Saccharomyces bayanis* and *Saccharomyces cerevisiae* yeasts can be distinguished. Experts identified sharp acidity in samples SB1 and SC4 and soft acidity characterized by lactic acidity in samples SB2 and SC3. Lactic acid is one of the major acids in ciders and succinic acid is the main acid produced by yeasts during the course of the alcoholic and glycerol-pyruvic fermentation, both present in Asturian natural ciders (Suarez and Picinelli, 2001).

Table 3. Cider flavours identified by experts

Class	General characterization	Characterization of subclasses	Identified characterization in juices samples			
			SB1	SB2	SC3	SC4
1	Sour, acidic	apple (sharp) acid	+	-	-	+
		vinegar	+	+	+	+
		lactic (soft) acid	-	+	+	-
		citrus sour	+	+	+	+
2	Aromatic, fragrant, fruity, floral	alcoholic (wine, fusel)	+	+	+	-
		solvent-like	-	+	+	-
		esters (apple-like)	+	+	+	+
		esters (pear like)	-	-	+	-
		citrus fruit	+	+	-	+
6	Soapy, fatty	fatty acid	+	-	+	-
7	Sulphury	sulphury, yeasty	+	-	-	+
8	Oxidised, stale,	stale, mouldy	+	+	+	-
10	Bitter	bitter	+	+	-	-
11	Mouthfeel	astringent	+	+	+	+
12	Fullness	body(satiating)	+	+	+	+

+ expert identified flavours in cider
– expert not identified flavours in cider

All samples were characterized with apple like esters and in sample SC3 also pear notes were identified. Alcohol, wine, fusel notes were identified in ciders SB1, SB2, and SC3.

Evaluation of the intensity of sensory properties

Significant differences ($p < 0.05$) among the yeasts for following evaluated sensory properties: clarity, yeast aroma, apple taste and sour taste (Fig.1) were observed. For clarity significantly higher results for cider SC4 was identified and also for this sample had higher intensity of yeast aroma. Significant differences ($p < 0.05$) among samples in apple taste and higher results show cider SC3, followed by SC4 and SB2. In previous investigations volatile compounds of ciders were analysed using solid phase microextraction followed by gas chromatography (Riekstina-Dolge et al., 2011).

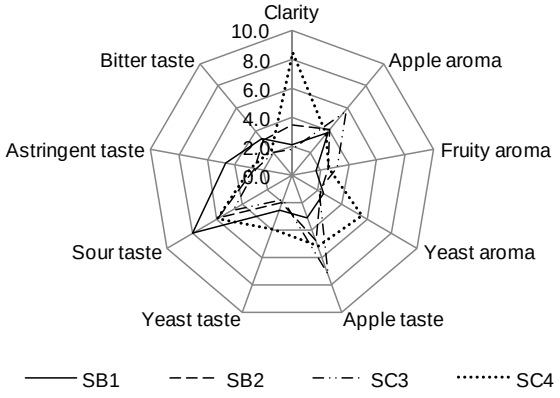

Figure 1. Spider plot for sensory properties of cider

2-methylbutyl acetate, hexyl acetate, hexan-1-ol was identified as main volatiles of apple juice. According to these results, correlation between content of volatile substances and evaluation of intensity of fruit aroma, apple taste and aroma were performed (Table 4).

Table 4. Correlation between sensory properties and content of volatile compounds

Parameters	2-methylbutyl acetate	Hexyl acetate	Hexan-1-ol	Sum of three compounds
Apple aroma	0.92	0.15	0.50	0.99
Fruit aroma	0.49	0.63	0.40	0.72
Apple taste	0.59	0.60	0.51	0.82

Experts evaluation of apple aroma intensity correlated very close with 2-methylbutyl acetate (r=0.92) and with sum of 2-methylbutyl acetate hexyl acetate hexan-1-ol content (r=0.99) in cider. Generally closer correlation between sum of three analyzed volatile components and sensory properties were observed.
Also correlation analysis was performed to determine interactions between different sensory properties. Moderate correlation ($p<0.001$) between yeast taste and yeast aroma (r=0.689), clarity and yeast aroma (r=0.688), clarity and yeast taste (0.555) were observed. Principal Component Analysis (PCA) with rotation was applied to all flavour terms data to obtain a more simplified view of the total flavours characters of the cider (Fig.2).

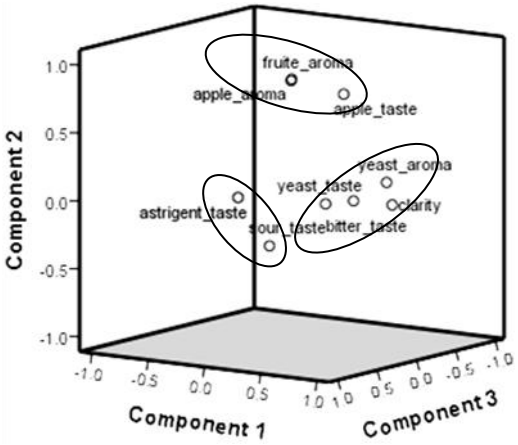

Figure 2. The diagram of factors by rotation

The first three PCs accounted for the highest variation, with 31.3, 23.5 and 12.1% out of total of 66.9 variations that could account for. The first factor contains characterising indicators as clarity, yeast aroma, yeast taste and bitter taste. In the second factor three parameters are included - apple aroma, apple taste and fruit taste. The third factor contains two descriptors - astringent and sour taste.

CONCLUSIONS

Selection of yeast is very important factor that influence final quality of cider. Four descriptors were significant for characterization of differences of ciders fermented by different yeasts, namely, sour taste, apple taste and yeast aroma and clarity. Apple taste and aroma, and also fruit aroma are important quality parameters of ciders and taking into account these evaluations as the best cider SC3 was observed.

ACKNOWLEDGEMENTS

The research has been done within the National Research Programme “Sustainable use of local resources (earth, food, and transport) – new products and technologies (Nat Res)” (2010.-2013.) project No. 3. „Sustainable use of local agricultural resources for development of high nutritive value food products (Food)” and ESF project “Support for the implementation of LLU doctoral studies” contract No 2009/0180/1DP/1.1.2.1.2/09/IPIA/VIAA/017. Authors also acknowledge Latvia State Institute of Fruit Growing for supply with apples.

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STRUCTURAL AND TEXTURAL PROPERTIES OF RICE-CARROT EXTRUDED PRODUCTS

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ABSTRACT: Extrusion cooking is used in the food industry, to produce direct expanded snacks; the nutritional value of these extruded snacks usually increases with the addition of high-value food ingredients. The objective of the present study was the determination of the effect of process conditions on the structural and textural properties of rice extrudates enriched with carrot. Extrudates were prepared using a twin-screw extruder, operated at different conditions, including screw speed (150-250 rpm), extrusion temperature (140-180 ºC), feed moisture content (14-20%) and carrot-rice flour ratio (5-10%). True density of the products was measured using a helium stereo-pycnometer and apparent density was obtained by measuring their dimensions with a caliper. The expansion ratio was determined as the ratio of the diameters of the extrudates to the die. The textural properties (maximum stress, maximum strain and elasticity modulus) of extruded products were obtained using a universal testing machine. A stereomicroscope was used in order to visualize the macrostructure of selected extrudates. Simple mathematical models were used in order to correlate the examined properties with the extrusion conditions. Apparent density increased with moisture content and material ratio and decreased with temperature and screw speed, while porosity presented the opposite trend. Expansion decreased with moisture content, material ratio and temperature, while a screw speed rise increased the expansion. Moisture content and extrusion temperature affected positively the textural properties. The increment of carrot concentration led to the increment of maximum stress and elasticity modulus and the decrement of maximum strain, whereas screw speed caused opposite trend.
Key words: *carrot, extrusion, modelling, rice flour, structural properties, textural properties*

INTRODUCTION

In recent years, there is an increasing trend for the consumption of high-value ready-to-eat products which preserve their nutritional characteristics. Among the processes which are used in the food industry, extrusion cooking is a continuous, high temperature-short time process, that produces direct expanded materials, with high quality (Ding et al., 2006). Food materials are cooked in a screw-barrel assembly by a combination of moisture, pressure and temperature in order to be mechanically sheared and shaped (Rodriguez-Miranda et al., 2011). Product quality depends on the extruder type, feed moisture, temperature profile, screw speed and feed rate (Thymi et al., 2005).

Nowadays, consumers have increased interest to the Mediterranean diet and the consumption of functional foods with high nutritional value. The nutritional value of the extruded snacks can be increased with the addition of high-value food ingredients, such as vegetables, which must be dehydrated in order to obtain the appropriate powder structure. Among the drying methods that are used in food industries, freeze drying is considered one of the most advanced methods, since it produces materials with high porosity, unchanged nutrition quality, superior taste, aroma, flavours and colour retention, as well as better rehydration properties (Krokida et al., 1998).

Structural properties, such as density and porosity, characterize the texture and quality of expanded products by controlling their taste and appearance, as well as they are indexes of the extent of puffing (Hagenimana et al., 2006). Expansion is also desirable for successful product development and is the primary quality parameter which determines the extrudate structure and texture. It is also associated with other important properties of the produced

snacks, such as crispness, water absorption, etc. (Chen and Yeh, 2001; Jyothi et al., 2009; Rodriguez-Miranda et al., 2011). In addition, the textural properties of foods are related with their sensorial characteristics, and finally with the quality and acceptance of the product by the consumers. The most common method in determining the texture of foods and especially of extruded products is compression (Mazumder et al., 2007; Vincent, 1998).
However the extrusion cooking is a widely studied process, the addition of high nutritional ingredients on the extrudates, has not been extensively examined. In addition, the determination of the effect of process conditions on the properties of extruded snacks is based on generalized models. As a result, the objective of the present study was the determination of the effect of process conditions and material characteristics on the structural and textural properties of extruded rice snacks enriched with carrot. Simple mathematical models were selected in order to predict structural and textural properties correlated with process conditions and material characteristics.

MATERIALS AND METHODS

Materials preparation and extrusion cooking

Rice flour was provided from Agrino Ev.Ge. Pistiolas S.A. and carrots were purchased from the local market. Carrots were preliminary dehydrated in a laboratory freeze dryer (Leybold-Heraeus GT 2A, Koln, Germany) for two days, and were ground to powder. Rice flour and carrot powder were mixed in different ratios, ranging from 5 to 10% (carrot concentration) and equilibrated to three moisture levels: 14, 17 and 20%. Extrusion was performed in a co-rotating double screw extruder (Prism Eurolab, model KX-16HC, Staffordshire, UK), consisting of five independent temperature control zones. The diameter of the die was 3 mm. Extrusion temperature varied from 140 to 180 ºC and the screw speed ranged from 150 to 250 rpm. Extruded materials were stored in laminated bags until required for analysis.

Measurement of structural properties

The true volume of the samples, ground to powder to remove most of the internal pores, was estimated using a helium stereopycnometer (Quantachrome multipycnometer MVP-1). The true density (ρ_{ts} (kg/m^3)) was expressed by the ratio of the mass of dry solids to the volume of dry solids. The extruded snacks were cut in cylinders and the apparent volume was obtained by measuring their actual geometric characteristics (diameter, length), using a digital Vernier caliper. The apparent density was determined using the equation:

$$\rho_{ap} = \frac{4 \cdot m_s}{\pi \cdot d^2 \cdot h} \quad (1)$$

where ρ_{ap} (kg/m^3) is the apparent density, m_s (kg) the mass of the extrudates, d (m) the diameter and h (m) the length of each extrudate.
The porosity, ε, was estimated using the equation:

$$\varepsilon = 1 - \frac{\rho_{ap}}{\rho_{ts}} \quad (2)$$

and the expansion ratio, *Exp*, was determined according to the equation:

$$Exp = \frac{d}{d_o} \quad (3)$$

where d (m) is the diameter of the extrudates, d_o (m) the diameter of the die.
The macrostructure of the samples was visualized using a stereomicroscope (Olympus, SZ). The samples were cut at a thickness of 2 mm and photographed at 10x magnification.

Measurement of textural properties
The textural properties of extrudates were obtained using a universal testing machine (Zwick model Z2.5/TN1S, Germany-Ulm). The uniaxial compression tests were performed at room temperature, using a 100 N load cell. Constant deformation rate of 5 mm/min was used for all the materials. Extrudates were cut in cylinders and their dimensions were measured, prior to each experiment, using a caliper. Force and deformation were recorded electronically and the resulting stress–strain compression curves were constructed according to the equations:

$$\sigma = \frac{F}{A} \tag{4}$$

$$\varepsilon_n = \frac{\Delta L}{L_o} \tag{5}$$

where σ (Pa) is the stress, ε_n (mm/mm) is the strain, A (m^2) is the cross section area, L_o (m) is the initial thickness of the samples, F (N) is the force and ΔL (m) is the deformation.

Mathematical modelling
A simple power model, containing parameters with physical meaning, has been selected in order to correlate the structural and textural properties with process conditions and materials characteristics, and is expressed with the equation:

$$I = I_o \left(\frac{X}{X_o}\right)^{i_X} \left(\frac{100-C}{100-C_o}\right)^{i_C} \left(\frac{T}{T_o}\right)^{i_T} \left(\frac{N}{N_o}\right)^{i_N} \tag{6}$$

where I is the measured property, X (% w.b.) the feed moisture content, C (%) the carrot concentration, T (ºC) the extrusion temperature, N (rpm) the screw speed, I_o, X_o, C_o, T_o, N_o, the corresponding values of the experimental parameters at reference conditions, i_X, i_C, i_T, i_N are parameters dependent on the materials.

Statistical Analysis
Regression analysis was used to estimate the models' parameters, as described by Maroulis et al. (1988). The analyses were performed using Statistica software (Statistica Release 7, Statsoft Inc, Tulsa, OK, USA). Significant differences were considered when $p<0.05$.

RESULTS AND DISCUSSION

The true density of all extrudates was taken as a constant equal to the density of the solid material. True density was estimated equal to 1480 kg/m^3 with a standard deviation of ±18 kg/m^3.
The apparent density of the extrudates increased with the increment of moisture content and concentration of carrot in the mixture. The increased moisture content of extruded products changes the molecular structure of amylopectin contained in starch, reduces the viscosity and increases the density. The increment of carrot concentration is characterized by high protein and fiber content and when it is added to starchy products, causes an increase in density, as expected from the literature (Sacchetti et al., 2004). The increment of extrusion temperature decreases the melt viscosity of the mixture, leading to bubble growth during extrusion and creating a less dense structure. The increment of screw speed caused the reduction in density of extrusion products. This phenomenon is attributed to the reduction of the viscosity, the increment of the elasticity of the dough and the gelatinization of starch. The

corresponding results are presented in Fig. 1, where solid lines represent the predicted values of apparent density. The calculated parameters of Eq. 6 are presented in Table 1.

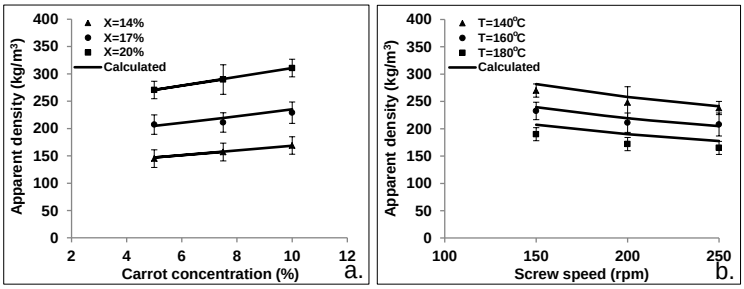

Figure 2. Effect of a) moisture content and carrot concentration, (*T*=160ºC, *N*=200 rpm) and b) extrusion temperature and screw speed, (*X*=17%, *C*=7.5%) on apparent density of extruded products

Table 1. Parameters estimation for structural and textural properties of extruded snacks.

	ρ_{ap}	*Exp*	σ_{max}	ε_{max}	*E*
I_o	219.330	2.394	161.683	0.243	605.609
i_X	1.712	-0.305	1.640	0.334	2.863
i_C	-2.555	1.981	-3.290	3.510	-3.812
i_T	-1.218	-0.770	2.702	0.378	1.953
i_N	-0.306	0.248	-0.541	0.412	-0.387

**for all the estimated parameters p<0.001*

As shown in Fig. 2, porosity presented the opposite trend, as expected, according to Eq. 2.

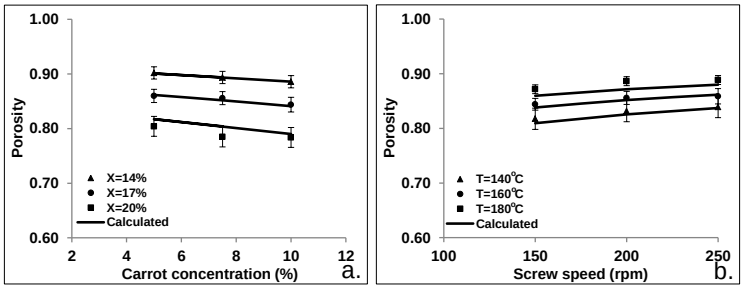

Figure 3. Effect of a) moisture content and carrot concentration, (*T*=160ºC, *N*=200 rpm) and b) extrusion temperature and screw speed, (*X*=17%, *C*=7.5%) on porosity of extruded products

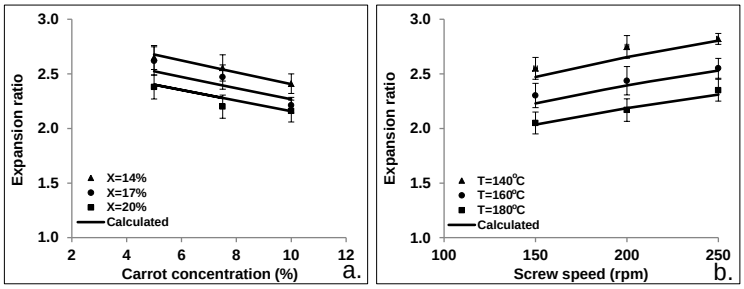

Figure 4. Effect of a) moisture content and carrot concentration, (*T*=160 ºC, *N*=200 rpm) and b) extrusion temperature and screw speed, (*X*=17%, *C*=7.5%) on expansion ratio of extruded products

Expansion of extrudates decreased with increasing moisture content, carrot concentration and temperature, while it increased with increasing screw speed, as shown in Fig. 3. The increment of moisture content reduces the viscosity, leading to more compact structures. Higher levels of carrot resulted in significant reduction of the expansion. The increased concentration of protein and fibres caused the formation of less expanded products due to the interaction between these components and starch. Fibres can also cause rupture of cell walls and prevent air bubbles to expand (Lue and Huff, 1991). The decrease in expansion with increasing temperature is possibly attributed to greater degradation of starch

(Hagenimana et al., 2006). The increase of screw speed caused an increase in expansion, due to the reduced viscosity and the increased elasticity of the dough. These observations were enhanced with stereomicroscopy. Fig. 4 illustrates that expansion ratio of extrudates decreased with increasing temperature, while it increased with increasing screw speed.

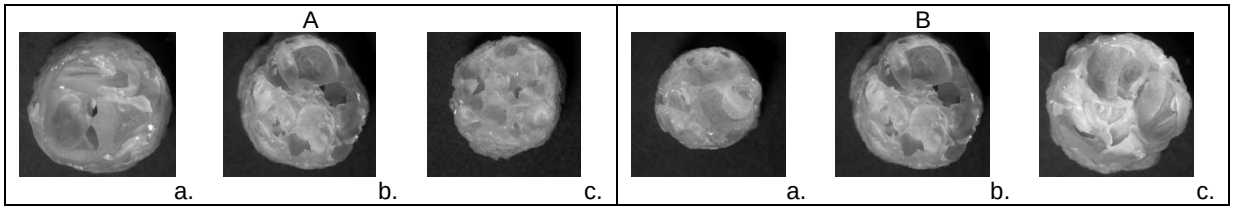

Figure 5. A) Effect of extrusion temperature on the structure of extruded products (*X*=17%, *C*=7.5%, *N*=200 rpm) a) *T*=140 ºC, b) *T*=160 ºC, c) *T*=180 ºC), B) Effect of screw speed on the structure of extruded products (*X*=17%, *C*=7.5%, *T*=160ºC) a) *N*=150 rpm, b) *N*=200 rpm, c) *N*=250 rpm

Fig. 5 presents a typical stress strain curve. Fig. 6 shows the effect of material characteristics on the maximum stress (σ_{max}), maximum strain (ε_{max}) and elasticity modulus (E) of extruded products. It can be observed that maximum stress and elasticity modulus increased with the increment of moisture content and carrot concentration, whereas maximum strain increased with feed moisture content and decreased with carrot concentration.

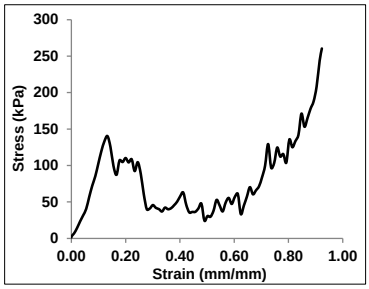

Figure 6. Typical stress strain curve

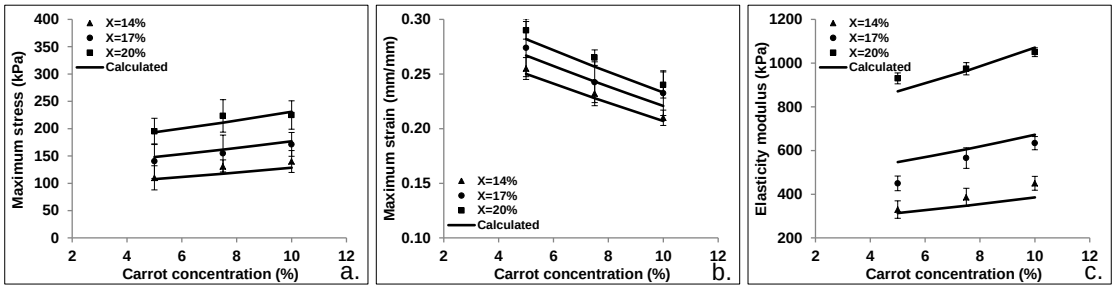

Figure 7. Effect of moisture content and carrot concentration, (*T*=160 ºC, *N*=200 rpm) on a) maximum stress, b) maximum strain and c) elasticity modulus of extruded products

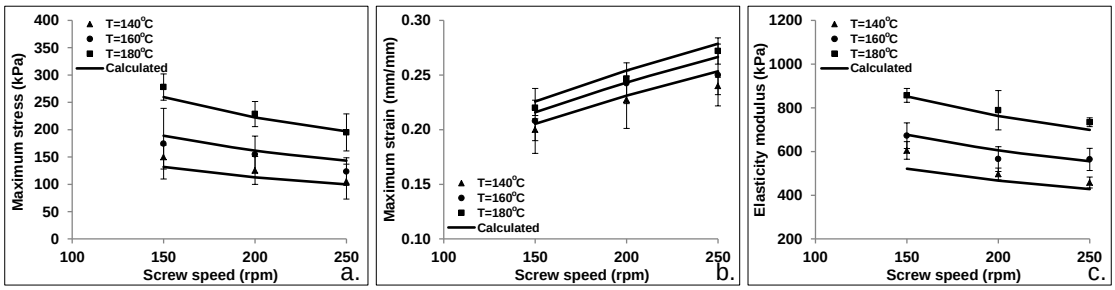

Figure 8. Effect of extrusion temperature and screw speed, (*X*=17%, *C*=7.5%) on a) maximum stress, b) maximum strain and c) elasticity modulus of extruded products

Fig. 7 shows the effect of process conditions on the textural properties of extrudates. Maximum stress and elasticity modulus decreased with screw speed and presented opposite

trend with porosity. The less dense extrudates presented lower values of maximum stress. The increment of stress with temperature, where the density of the products is reduced, may be attributed to the formation of more solid cell walls due to the high temperature (Agbisit et al., 2007). Maximum strain increased with temperature and screw speed.

CONCLUSIONS

Extrusion conditions affected the structural and textural properties of the extruded rice snacks enriched with freeze dried carrot powder. Apparent density increased significantly with moisture content and material ratio and decreased with temperature and screw speed, while the opposite trend was observed for porosity. The extrudate expansion decreased as the moisture content, material ratio and temperature increased, while a screw speed rise resulted in products with higher expansion ratio. The above-mentioned results were enhanced with the images obtained from the stereomicroscope. The textural properties were also significantly affected by process conditions. The denser extrudates presented higher values of maximum stress and elasticity modulus. The most desirable extruded products with higher expansion ratio were those contained 14% initial moisture content and were produced at medium temperatures. Information about the structure and texture of foods is very useful for the process design and the production of high-value foods with desirable properties.

ACKNOWLEDGEMENTS

The authors would like to thank the Research Committee of the National Technical University of Athens (NTUA) and the Alexander S. Onassis Public Benefit Foundation for their financial support. We would also like to thank Agrino Ev.Ge. Pistiolas S.A for providing rice flour.

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EDUCATION IN SENSORY SCIENCE IN SLOVENIA

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ABSTRACT: Sensory analysis is an indispensable part of the development, characterisation and quality control process, respectively. It is a scientific discipline applying principles of experimental design and statistical analysis. The need for education in sensory analysis of food was recognized in Slovenia as early as 1975. The courses in sensory analysis became a part of B.Sc. and post-graduate study programmes at Department of Food Science and Technology of Biotechnical faculty. Sensory courses and trainings are also organized for various audiences i.e. beekeepers, school teachers, farmers, consultants and winemakers. The majority of Slovenian food producers nowadays employ internal sensory assessment of products as a part of quality control. At the Department panels of sensory assessors were formed, whose members collaborate with food industry in product developments and quality controls as well as they participate as lecturers in sensory courses and expert assessors in sensory evaluations of products for various clients. The importance of sensory quality of food was also found important for consumer protection and is therefore applied in quality testing of food products performed in collaboration with Slovenian consumer association.
Key words: *sensory analysis, education, food, assessors, consumers*

INTRODUCTION

Sensory evaluation is preformed instinctively every day. Humans have used their senses to discriminate between good and spoiled, safe and unsafe foods for several thousands of years. Over the centuries various methods of food assessment were developed, incited by early trade (Drake et al., 2009). In Slovenia, we have about two hundred years old documented source of sensory evaluation, a cook book, in which the author wrote about the smell, colour and taste as parameters of water quality as well as they are important indicators of the quality of food in relation to human health (Vodnik, 1834).

Although professional tasters and consultants were employed in food and beverage industry since the early 1900s, the field of sensory evaluation advanced rapidly not earlier than with the expansion of processed foods in the second half of the 20th century (Lawless and Heymann, 1998; Meilgaard et al., 1999). In Scandinavian countries a triangle test, a difference test method, was developed in about 1940, while in other European countries modern sensory analysis was employed not until 1950. Although the first book on sensory analysis was written in Polish in 1957 by Tilgner, and the second one by Japanese authors in 1962 (Jellinek, 1985), the definition of sensory evaluation, as cited nowadays, was determined in 1985. Sensory evaluation has been defined as a scientific discipline used to evoke, measure, analyse, and interpret responses to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch and hearing (Stone and Sidel, 1985). As such, it enables determination of the sensory properties of a food product and provides noteworthy information about sensory characteristics of the product to food technologist and to others involved in the product development and sales (Lawless and Heymann, 1998). Sensory analysis is nowadays indispensable in food industry. It is employed in a development of a new product, as well as in the quality control process. However an early survey revealed that food producers often utilized inappropriate test methods in the development of their product, as they were confused about the purpose of the chosen method and the type of information such method provided (Brandt and Arnold, 1977).

Biotechnical faculty of University of Ljubljana has been running courses in food science and technology since 1961. At the time when the first generations of students graduated and

started their professional careers in the food industry, various studies from the field of sensory analysis have already been conducted abroad and results published. However the accessibility of professional and scientific journals was quite limited at that time and language constraints were often present. The first "*Živilski dnevi*" (engl. *Food Days*), a form of postgraduate education for food technologists, organized in 1975 and dedicated to sensory analysis of food, is considered as the beginning of systematic work in this field in Slovenia. Until then there wasn't any professional literature available in Slovenian language, neither was sensory evaluation methodically introduced into the process of evaluation of food quality in our country. Presented topics published as papers in the proceedings comprised did not only broaden the aspect of food quality and opened new possibilities in product development, but also introduced the Slovenian vocabulary in sensory analysis of food (Bučar, 1975).

METHODS AND MATERIALS

Sensory analysis is used for different purposes of a food product evaluation. It is used to establish difference among similar products, or to characterize and measure sensory properties of a product. Moreover, it is used to ascertain whether product differences are acceptable or noticeable to consumers.

Sensory assessors

A group of sensory assessors, i.e. a sensory panel is consisted of individual members of the panel. Similarly as it is important to calibrate an apparatus, an instrument, it is necessary to properly select, train and monitor the performance of the panel members, since the results of the sensory analysis depend on them. A sensory assessor is a person, who is involved in sensory evaluation, irrespective of the competence and qualifications obtained. Regarding their abilities of perception, discrimination, training level and experience, sensory evaluation is carried out by three types of assessors (ISO 8586-1:1993; ISO 8586-2:2008):

1. Naïve – those who have had no training or beginners who have only been introduced in sensory methodology;
2. Selected – have been screened for their abilities, selected and trained for sensory evaluation by a certain method, and to work in a specific field (e.g. evaluation of dairy products);
3. Expert – assessors who have undergone an extensive training and are able to reliably detect and measure sensory properties of a food product.

The process of selection and training of assessors is comprised of several stages:

- recruiting and preliminary selection of the candidates, naïve assessors,
- training of the novices (naïve assessors),
- selection of the novice regarding their abilities and performance in certain sensory tests,
- selection of candidates, who have passed the previous stage, with regard to their ability of discrimination, memorizing and expression of perceived sensory stimuli (for descriptive sensory analysis),
- further education and training of candidates, who have fulfilled requirements in all stages of the selection of assessors.

Sensory methods

Different sensory methods are applied in the evaluation of sensory characteristics of foods. The sensory tests used are divided into two main types:

1. Affective tests that are used for determination of preference or level of acceptability. The questions raised in these tests are "How pleasant is the product?", "Which of the two products do you prefer?" The attributes evaluated by these tests are preference, suitability and image of the products.
2. Analytical tests that are applied to identify differences in sensory properties and to measure selected property. The methodologies used are rigorous in order to minimise

external influences and assure objective, precise and reproducible descriptions of sensory properties. These tests are further classified into:

- Discrimination tests, which allow detection and determination of small differences between the two products.
- Tests using scales for classifying samples into categories or classes, or to determine the intensity of an attribute or of the difference between the products (ISO 4121:2003; ISO 8587:2006).
- Descriptive analysis, which is performed only by a panel of experts. The result is a complete description of all perceived sensory properties in the order as they are perceived (ISO 11035:1994; ISO 11036:1994).

Education

Education in sensory analysis, which is carried out for students of Biotechnical faculty and of other schools as well as for participants of various seminars, is based on today already reach and diverse literature (Jellinek, 1985; Lawless and Heymann, 1998; Piggott, 1988) and applying all available ISO standards. The later give general guidance on the use of sensory analysis (ISO 6658:2008), for the design of test rooms (ISO 8589:2007), for selection, training and monitoring of different types of assessors (ISO 8586-1:1993; ISO 8586-2:2008) as well as on the implementation of various sensory methods.

Different sensory methods are used for education and training in sensory analysis. The number and types of tests applied depend on the purpose of the education, on the candidates as well as on the nature of the samples presented:

- *Discrimination tests* befit the education and training process and are quite easy to carry out. They are used to train the candidates in detecting the difference between the two samples, when the difference is very small or non-existent. Among the five discrimination tests: Paired comparison test (ISO 5495:2005), Triangle test (ISO 4120:2004), Duo trio test (ISO 10399:2004), Two out of five test and “A” - “not A” (ISO 8588:1987) the first two are the most suitable and commonly used. The Paired comparison test is the most simple and rapid test, while the Triangle test is the most sensitive one.
- During the training candidates are familiarized with the importance of different *scales* (nominal, ordinal, interval, and ratio scales) and their types: category scales, line marking scales, magnitude-estimation scales (Lawless and Heymann, 1998). The scales are distinguished in four groups on the basis of the ordering and distance properties inherent in measurement rules: classification, ranking, measuring magnitudes, assuming equal distances between points on the scale, and measuring magnitudes, assuming equality of ratios between points (Stone and Sidel, 1985). In ISO 6658:2005 four different types of measurement with scales are distinguished: classification, grading, ranking, and rating and scoring. Ranking and rating with scales are the most often used in the process of schooling; in ranking candidates have to rank the samples on the scale in order of intensity of particular property or liking, respectively, while in rating samples are allocated on an ordinal scale in a similar way, but more than one sample may be allocated to the same scale point.
- Candidates following seminars of more than 30 school hours are introduced also into the methods of *descriptive analysis*. These are the most sophisticated sensory techniques that can be carried out only by trained assessors. Candidates learn about how descriptors are created and sensory properties determined, about principles for setting the criteria for assessment of the properties.

Education in frames of study programmes of Biotechnical Faculty

Extent of courses from sensory analysis depends on the level of study programme, i.e. Bologna first (BSc) or second level (MSc), respectively. The courses vary in hours, in profoundness of theoretical knowledge and in activities performed by students. We have published a textbook *Sensory analysis of food* (Golob et al., 2006) in mother tongue to bring chapters from sensory analysis to students and other trainees.

Students of 2nd year of academic study programme in Food science and nutrition (BSc) are introduced into basics of sensory evaluation of food (8 hours of lectures) and carry out some

of the screening tests (6 hours of exercises) in the frames of the compulsory subject Food quality and legislation.
In depth knowledge of the physiology of perception, the cellular mechanisms of perception, about individual sense organs and senses, on perception of colour, flavour, sensory active components, texture, off odours and foreign aroma, as well as on the E-nose, GC-olfactometry, tests with consumers, and methods of data analysis applying tools like MS Excel or SPSS are provided through both, an elective subject Sensory evaluation techniques for BSc (35 hours of lectures, 15 hours of seminars, 20 hours of exercises) and a compulsory subject Sensory analysis for MSc students (25 hours of lectures, 10 hours of seminars, 15 hours of exercises). Examples of software solutions for sensory analysis and data collection are provided to students; although only through the demonstration by the lecturer. Besides a large number of tests that students carry out during practical exercises, they are included also in preparation of samples, sample labelling, preparation of evaluation forms, and realization of sensory evaluation itself. Seminars for MSc students comprise elaboration of the project involving selection of the issue, a food product to evaluate, and adequate sensory method(s), design of evaluation forms, execution of the evaluation process with a sensory panel, data analysis and critically commenting of the results. Students submit written seminar and present their project to audience.
Students of Interdisciplinary doctoral study programme in biosciences at 3rd Bologna level can attend an elective subject Linkage of sensoric and instrumental methods, in which contemporary sensory and instrumental techniques in food processing and quality control are discussed and holistic approach in evaluation of quality is stressed.

Education for end users
Education in the context of seminars is composed from theoretical and practical part, i.e. lectures and practical exercises, usually in proportion 2:3 or 3:4, respectively. The amount of seminar hours varies depending on the requirements of the trainees and level of knowledge comprehended. Seminars of 15 hours or less are intended for those, who anticipate only basics from sensory evaluation, while over 30-hour, 50-hour or even 75-hour seminars are designed for trainees, who wish to be trained and qualified for the sensory evaluation in a specific field. Seminars are organized for differently sized groups, usually with minimally 10 and up to 40 participants. For practical exercises trainees are arranged into groups - panels of 10.
Most of the attendees have only scarce or none prior knowledge from the sensory analysis, therefore lectures comprise general theoretical knowledge from sensory science: definition of sensory analysis, general conditions for sensory analysis, types and training of sensory assessors, physiological basis of perception, and sensory methods - various tests, their applicability, advantages and disadvantages. Practical work – exercises include a) tests for evaluation of sensory sensitivity of the candidates using model systems: identification of four basic tastes, the ability to memorize the intensity of a stimulus, the ability of smelling, and b) different sensory methods used in practice: discrimination tests, scales etc. on model systems and food products. For assessments of the samples mainly paper forms are used, while samples of meat and meat products are evaluated also by using tailored computer programme. The attendees to the seminar receive a compilation of lecture notes, a study material that is more or less extensive, depending on the type and purpose of the education.

RESULTS AND DISCUSSION

Many graduates in Food Science and Nutrition are involved in sensory evaluation also in their professional career. Those working as lecturers in secondary schools and vocational colleges, have introduced chapters from sensory science into school curricula, while food technologist working in the industry apply sensory evaluation in a routine quality control or when modifying an existing or developing a new product. At the later they are often confronted with issues of acceptability or liking of such product among consumers. On the account of good cooperation between the industry and the faculty, many of these questions

may be solved by tests with consumer panels consisting of students, while at the same time the students experience the real life problems and professional challenges.
However in-depth knowledge of sensory evaluation is often needed by professionals from the food industry, therefore we organize different short courses and post-graduate seminars adjusted to users' demands. Sensory courses (seminars) and trainings are also organized for various audiences i.e. beekeepers, school teachers, farmers, consultants and winemakers.
The lectures and exercises are tailored to the purpose and scope of the seminar. Education of sensory assessors of a particular food product, like **honey assessors**, involves also topics from beekeeping technology, honey production and processing, European honey legislation, the rules on honey sensory assessment in Slovenia and presentation of the assortment of honeys produced in Slovenia. Practical exercises comprise, in addition to the aforementioned tests for screening and training of candidates, an extensive training on a given product, in this case, honey. A selection of typical samples is presented to attendees to learn about recognition of sensory properties and about their characteristics (e.g. intensity). Later candidates encounter a variety of samples of particular type to evaluate the intensity of their sensory properties, detect possible defects, and discuss the findings.
It is important for each profile of participants, nature and scope of the seminar that adequate samples are provided for the training and the assessment of the performance of trainees. Candidates have to fulfil two types of conditions to qualify for sensory assessor of a given food group as summarized in Table 1. Conclusion of the seminars is usually associated with a festive award ceremony.

Table 1. Minimum criteria for obtaining a license *Sensory assessor* of a particular food product or group of products.

1. Sensory tests	**2. Assessment of food samples**
- Thresholds recognition test - Recognition of basic tastes test - Intensity memory test - Discrimination test - Ranking test - The minimum number of total points is defined in advance	- Evaluation of sensory properties of a number of samples from the particular food group using defined sensory methods - Repeatability of the assessment results of a trainee - Assessments of a trainee must be placed within the set criteria. - The number of samples is defined in advance.

Trainees, licensed sensory assessors, may participate in sensory panels of sensory contests and assessment at different levels: local, regional and international. However to take part in sensory panels the continuity of work and training is required, as well as license renewal within the prescribed time. The license expires also when the candidate does not participate in sensory evaluation for more than two years. For example **assessors of wine, must and other products from grape and wine** have to attend several sensory evaluations annually and an additional training every two years. The training of this profile of sensory assessors was founded by Wine act (Wine act, 2006) and has been running in Slovenia for 8 years. In this time 574 candidates have been trained. An overview of different profiles of sensory assessors trained at Biotechnical faculty is presented in Table 2.

Table 2. Profiles of sensory assessors, number of candidates and course duration.

Type of sensory assessors or attendees	Number of trained attendees	Course duration (hours)
Assessor of wine, must and other products from grape and wine	574	74
Honey assessor	60	50
Honey inspector	150	27
Beekeeping inspector	43	16
Assessor of meat and meat products	331	20
Official inspectors	10	16
Agricultural advisors	38	9
Teachers – basics course	41	16
– advanced course	12	8
Paper industry	10	8
Food industry – basics course	36	16
– advanced course	21	16

At the Department of Food Science and Technology panels of expert sensory assessors were formed, whose members collaborate with food industry in product developments and quality controls. With their professional work these assessors contribute to the characterization and better recognition of typical Slovenian food products. Moreover, they are involved as lecturer in sensory seminars and as panel members in sensory evaluations of food products for various clients, among other Slovenian consumer association, or at different contests and assessments. In accordance to the requirements for training of sensory assessors the sensitivity and performance of panel members is being tested regularly. Besides, they improve their skills and proficiency by attending international conferences on sensory science and by participating in international sensory panels.

CONCLUSION

The majority of Slovenian food producers nowadays employ internal sensory assessment of products as a part of quality control. Generally, sensory analysis is in Slovenia well recognised among professionals. As it was observed, the quality of food products was improved over the years of continuous education services, and the progress was notable especially in products of artisanal production, e.g. honey. Recently the club of honey assessors, and wine assessors, respectively, was established, that concerns about promotion of Slovenian honey and wine, respectively, with emphasis on sensory characteristics of these products. However the education curriculum needs to be further enriched with topics on consumer science. Moreover, implementation or diffusion pathways of information obtained with sensory analysis to the end users should be clarified. Only so, the importance of continuous training of the assessors will be recognised and understanding of differences between sensory evaluation and hedonic tasting by the general public improved.

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INFLUENCE OF FOOD VISCOSITY ON THE PERCEPTION OF AROMA COMPOUNDS

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ABSTRACT: Food industry invests great effort into attempting to enhance aroma of the food products and often use natural and artificial flavourings. Aroma compounds are released from food during eating. Delivering aroma compound to olfactory and oral cavity (orthonasal and retronasal) is influenced by numerous factors. The aim of this research was to examine the influence of food matrix viscosity on aroma release. The orthonasally and retronasally intensity of four aroma compounds: ethyl butyrate, ethyl caproate, isoamyl acetate, and benzaldehyde which were added in the same amount in the four different food matrixes was sensory analysed by the fifteen trained panellist. The food matrix of different viscosity served as a barrier to the movement of a flavour substance. Food matrixes were represented by aqueous solution of sucrose, starch, oil-water emulsion and yoghurt, all in three levels of viscosity: low, medium, high. Viscosity was measured by MYR rotational viscometer. ANOVA and Principal Component Analysis (PCA) were used to analyse sensory results. It was found that the viscosity of sugar, starch and oil matrixes influence aroma release, while the viscosity of yogurt did not significantly influence on delivering aroma to human sensory receptor. Sucrose enhanced perception of aroma compounds. Transmission of aroma compounds to the oral cavity and olfactory epithelia from food matrixes was hindered.
Key words: *aroma compounds, peception, viscosity, sensory analysis*

INTRODUCTION

Compounds responsible for odours are designated as aroma compounds. Aroma compounds perceived on olfactory epithelium, results in odour character of food. Aroma of most food products consists of complex mixtures, sometimes consisting of several hundred compounds. Those compounds are volatile and the amount of volatile present in food is extremely low. More than 7000 volatile compounds have been identified in food. But of all volatile compounds only a limited number are important for aroma. Those compounds are called aroma active compounds. Intensity of aroma active compounds depends on their concentration and sensory threshold. Contribution of aroma active compounds to overall aroma perception is evaluated on the basis odour active value (OAV). It is ratio of aroma compounds concentrations in food to odour threshold (Leitner and Siegmund, 2009).Thus the presence, contents and composition of volatile substances in food have a substantial influence on its quality.The growth in the identification of aroma chompounds over the past five decades was a direct result of the development of new analytical techniques. Despite the advances in instrumental analysis, human nose is often more sensitive than any instrumental detector (Falcao et al., 2008). For aroma compounds to be perceived by human, they must be released from the food matrix so they can enter the airways of the nose and come into contact with the olfactory receptors. Aroma release from food depend on many factors: physiochemical characteristic aroma compounds themselves and their concentrations (2000; Bult et al., 2002; Plotto et al., 2008) physical state of matrix (Cook et al., 2003; Jaros, 2003; Seuvre at al., 2004) aroma compounds interactions with basic food components (Saint-Eve et al., 2006; Lawrence et al., 2009; Merabtine et al., 2010; Nasri et al., 2011) and the psychophysiology state of human (Grosch, 2000; Djordjevic et al., 2004; Ruijschop et al., 2009). Foods are a complex mixture of water, carbohydrates, lipids, proteins and other organic compounds which can interact with aroma compounds. The nature of these interactions can be chemical binding (irreversible covalent bonds), physicochemical

binding (van der Waals forces, hydrogen bonds, hydrophobic interactions or ionic bonds) or simply, the differing propensities for aroma compounds to be absorbed into different phases of the food. Physical considerations include those where the food matrix physically interferes with aroma a compound reaching sensory receptors, the food matrix serves as a barrier to the movement of an aroma compounds. The barrier may be small such as a food having some viscosity that reduces mixing or the surface area of food in the mouth lowering the possibility of evaporation in oral cavity or in air over food. The barrier may be very large, for very viscous food where aroma compounds remain locked in food matrix.
Therefore, the aim of this work was to sensory evaluate influence of food viscosity on aroma compounds release from different food matrix by trained panel, i.e to investigate influences of food viscosity on aroma delivering to oral cavity and olfactory epithelia.

MATERIAL AND METHODS

Samples and sample preparation

In this study four aroma-active componuds were used (Tab. 1).

Table 1. Basic data of investigated aroma-active compounds

Compound	Producer	Pure%	Treshold	According to author
Ethyl Butyrate (EB)	Acros Organics	99	1 µg/L	Takeoka et al., 1990
Ethyl Caproate (EC)	Acros Organics	99	1 µg/L	Takeoka et al., 1990
Iso-amyl Acetate (IAA)	Fisher Scientific	99.55	2 µg/L	Takeoka et al., 1989
Benzaldehyde(BA)	Panreac Sintesis	99	0.35 µg/L	Buttery et al., 1990

Series of 5 different concentrations of each aroma compounds were prepared for the training of the panellists. The first concentration in series was threshold value and each next was 50% higher than the previous one. Dilution was made with redistilled water. Concentration values were marked from 1 to 5 corresponding to odour active value OAV in order to simplify aroma intensity measure. Concentration of aroma compounds and their OAV are showed in Tab.2.

Table 2. Concentrations of aroma compounds in series (µg/L)

Compound	Odour active value (OAV)				
	1	2	3	4	5
EB and EC	1	1.5	2.25	3.375	5.0625
IAA	2	3	4.5	6.75	10.125
BA	0.35	0.525	0.7875	1.18125	1.771875

Table 3. Levels of viscosity solution measured by MYR viscometer (V1-R)

Food matrix Temp. 20 ^{0}C	Low viscosity (I)		Medium viscosity (II)		High viscosity (III)	
Sugar (sucrose)	Spindle/rpm	mPa s	Spindle/ rpm	mPa s	Spindle/ rpm	mPa s
Oil* (sunflower oil)	R2/100	125	R2/100	157	R2/200	520
Yoghurt	R2/100	99	#	#	R2/200	136
	R2/50	60	R2/200	107	R2/200	700
Starch paste	R3/100	350	R3/100	450	R4/100	680

**Oil matrix was presented just in two modality – low and high viscosity*

Food matrixes were presented with four modalities: sugar, (aqueous solution of sucrose) oil (commercial sunflower oil and emulsion of oil and water 50:50 with lecithin as emulsifier) protein (commercial yoghurt) and carbohydrate (maize starch paste). Each of food matrix was presented in three levels of consistency (adjusted by water) measured by MYR rotational viscometer marked as presented in Tab. 3.
Samples were prepared as follows: 50 millilitres of each food matrix in three levels of viscosity were put in 100 ml cup coded with a random 3-digit number. Cups were closed.

Just before a sensory evaluation in cups were added 5 ml of aroma compounds in concentration which corresponding to OAV-3 (see Tab 2).

Panellists
An established and trained panel of 15 panellists (7 male, 8 female) aged between 20 and 45 years, was used in the study. The panellists had been recruited and selected among 24 volunteers, on the basis of their sensory acuity, in particular their ability to distinguish between concentrations of the same stimulus. The panellists participated in six 1-h trainings and three 30-min evaluation sessions on different days over 6 week. During an initial training session panellists received specific training in the use of threshold concentration of stimuli and rated 5 different concentrations of the same aroma compounds.

Sensory analysis
Intensity of aroma compounds was evaluated orthonasal and retronasal. Panellists were offered a block of samples consisted of 4 series for each food matrix in same concentration (OAV 3 - Tab 2) of each aroma compounds in 3 different food viscosity. That means 4 blocks of food matrix x 3 levels of viscosity x 4 aroma compounds, in total 48 samples for each panellist. The panellists were asked to evaluate perceived aroma intensity firstly orthnasally and then retronasally. After they finished one block of samples they had pause of 10 min and then started with evaluations of other blocks of samples. Perceived aroma compounds intensity was quantified using numerical scale from 1 to 5. Due to easier using, the end points of scale were offered to panellist too where 1 was threshold concentration and 5 was the maximum concentration of tested aroma compounds in redistilled water (see Tab. 2).The panellists were asked to evaluate perceived aroma intensity of samples rated them between end points of scale. To avoid confusion the term ‘‘odour’’ was used to refer to the orthonasal olfactory perception evaluated above the cup and ‘‘aroma’’ to refer to the retronasal olfactory perception.

Data Analysis
Two-way ANOVA and, where necessary, Tukey multiple-comparison tests (α 0.05) were applied to the replicate data (n=15). For the visualizations of all experimental variables Principal Component Analysis (PCA) was performed. The aim of PCA was to determine the differentiations between food matrixes, their viscosity, as well as to distinguish between perceived aroma orhonasally and retronassally. All statistic analyses were performed using the statistical package StatBox 6.7 –Grimmerlow, Paris, France.

RESULTS AND DISCUSSION

Aroma release from the sweet matrix
To determine whether level of viscosity of sucrose solution had influence on odour perception for the 4 aroma compounds two-way ANOVA was performed.The ANOVA showed significant effect of the matrix viscosity ($F_{3.054}$= 3.8488; p=0.023357) and type of aroma compounds ($F_{2.663}$=4.3151; p=0.00592) on perceived intensity of odour. Tukey’s test ($p < 0.05$) showed a significant decrease of odour release with level of sucrose solution viscosity from low to high (Fig 1.A). Sucrose enhances intensity of odour released from sweet matrix for each tested aroma compounds (Fig 1.B). All compounds were scored above 3, what is concentration which corresponds to their OAV in water. Those results are in accordance with Labbe et al., 2007; who have concluded that sucrose enhanced odour and aroma.

Aroma release from the oil matrix
Intensity ratings for perceived odour decreased as a function of oil concentration (Fig. 2.A). Statistical analysis revealed a highly significant effect of the level of emulsion viscosity ($F_{3.932}$ = 12.396, p<0.001). However, there is no difference in perceived odour intensity of 4 tested aroma compounds as reported in Fig. 2.B. The odour intensity of each of four aroma

compounds in oil emulsion was below 3 means lower than theirs intensity in aqueous solution. It is well-known that most of aroma compounds are lipophilic. Therefore, in oil emulsion aroma compounds prefer to reside in the particulate oil phase and its concentrations in the continuous phase is decreased greatly thereby lowering its concentration in air phase. Investigation of Van Rut et al., 2002 showed that eighteen out of the 20 aroma compounds showed a significant change in air/liquid partition coefficient and therefore on odour release above liquid as results of change in emulsifier concentration.

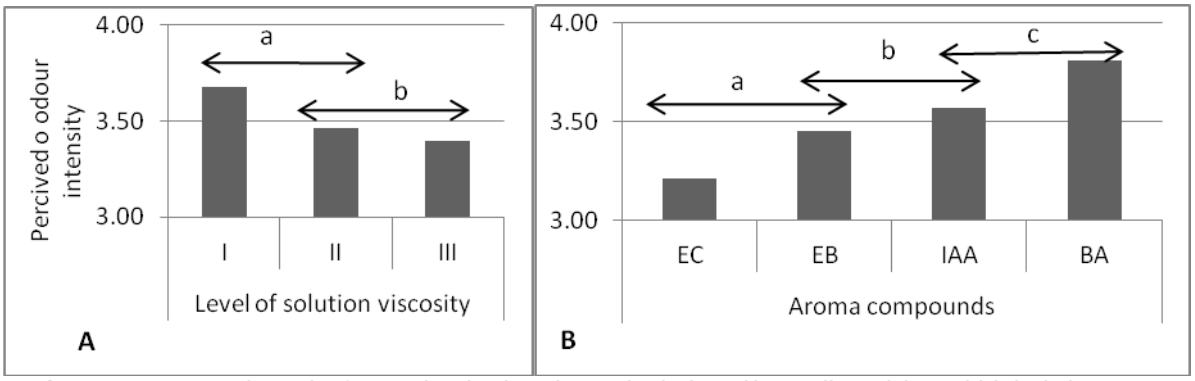

Figure 1.A Mean (n=15) of perceived odour intensity in low *(I),* medium *(II),* and high *(III),* sucrose solution viscosity. **1.B** Mean of perceived odour intensity of the individual aroma compounds *(EC-Ethyl Caproate EB- Ethyl Butyrate IAA- Iso-amyl Acetate, BA- Benzaldehyde)* The different letters indicate subset of significantly different samples obtained from Tukey test ($p \leq 0.05$)

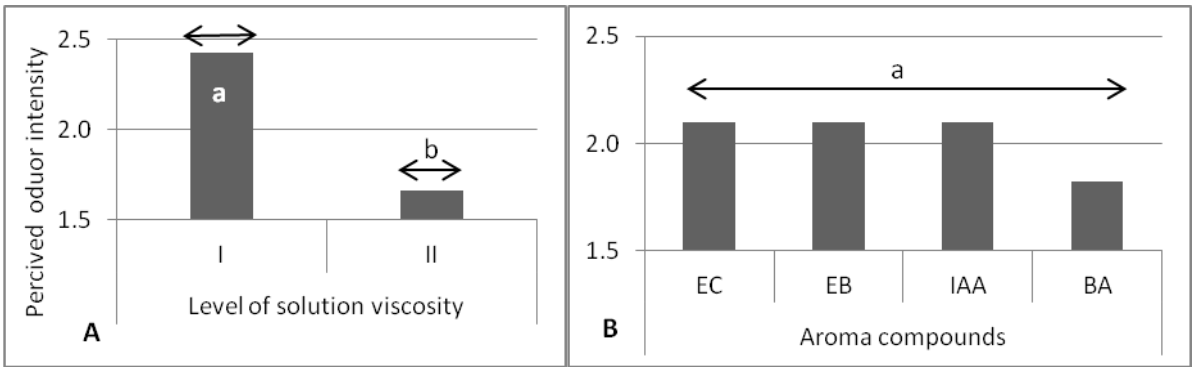

Figure 2.A Mean (n=15) of perceived odour intensity in low *(I),* and high *(III)* oil emulsion viscosity. **2.B** Mean of perceived odour intensity of the individual aroma compounds *(EC-Ethyl Caproate EB- Ethyl Butyrate IAA- Iso-amyl Acetate, BA- Benzaldehyde)* The different letters indicate subset of significantly different samples obtained from Tukey test ($p \leq 0.05$)

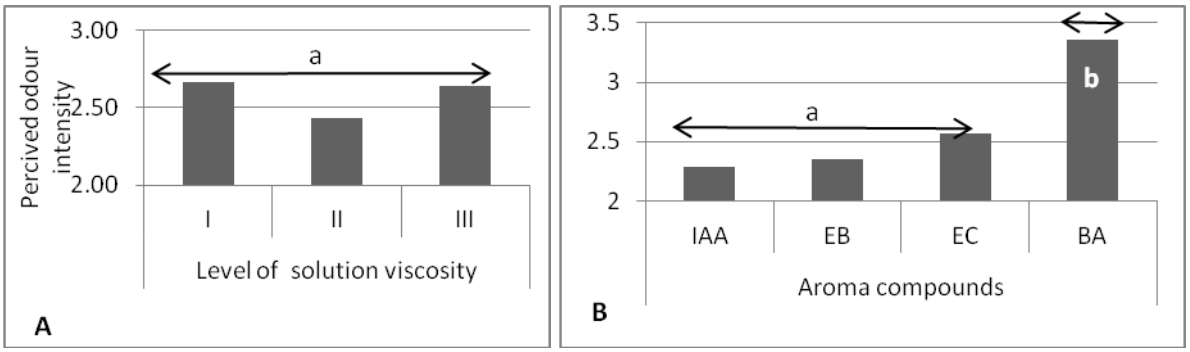

Figure 3.A Mean (n=15) of perceived odour intensity in low *(I),* medium *(II),* and high *(III),*yoghurt mat6rix viscosity. **3.B** Mean of perceived odour intensity of the individual aroma compounds *(EC-Ethyl Caproate EB- Ethyl Butyrate IAA- Iso-amyl Acetate, BA- Benzaldehyde)* The different letters indicate subsets of significantly different samples obtained from Tukey test ($p \leq 0.05$)

Aroma release from yoghurt

Differences in yoghurt viscosity did not influenceon intensity of aroma compound release significantly (Fig. 3.A). Unlike other compounds Benzaldehyde showed significantly higher perceived odour intensity. Odour intensity of Ethyl Caproate, Ethyl Butyrate and Iso-amyl Acetate in yoghurt were lower than 3 means protein as main component of yoghurt entrapment those aroma compounds.

Aroma release from starch matrix

Intensity ratings for perceived odour decreased as a function of viscosity of starch paste (Fig. 4.A). ANOVA revealed a highly significant effect of the level of starch viscosity ($F_{3.054} = 5.193$, $p < 0.001$) and kind of aroma compounds ($F_{2.663} = 4.3151$; $p<0.005$) on perceived intensity of odour. Tukey's test ($p < 0.05$) showed significantly higher perceived odour intensity from low then from medium and high viscous starch paste. Tukey's test ($p < 0.05$) showed significantly higher odour intensity for Benzaldehyd compared to other aroma compounds (Fig.4.B). It could be noticed that starch matrix reduced the release aroma compounds because all of them were assessed below 3 what is their OAV in aqueous solution.

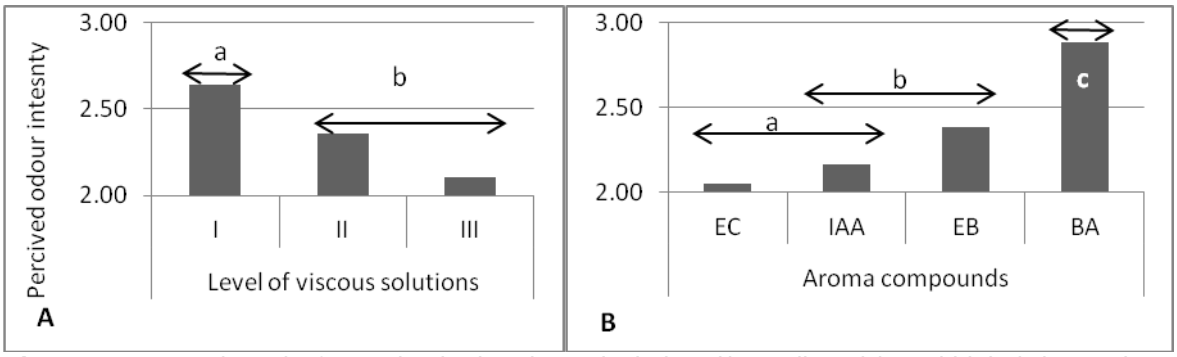

Figure 4.A Mean (n=15) of perceived odour intensity in low *(I),* medium *(II),* and high *(III),* starch paste viscosity.**4. B** Mean of perceived odor intensityof the individual aroma compounds *(EC-Ethyl Caproate EB- Ethyl Butyrate IAA- Iso-amyl Acetate, BA- Benzaldehyde)* The different letters indicate subsets of significantly different samples obtained from Tukey test ($p \leq 0.05$)

PCA analysis

In order to get an overview of the similarities and differences among 22 samples on the basis of food matrix (sugar, oil, starch, yoghurt) levels of matrix viscosity (I, II, III) and way of perceived intensity of aroma compounds (odor, aroma) principal component analysis was performed (Fig 5).

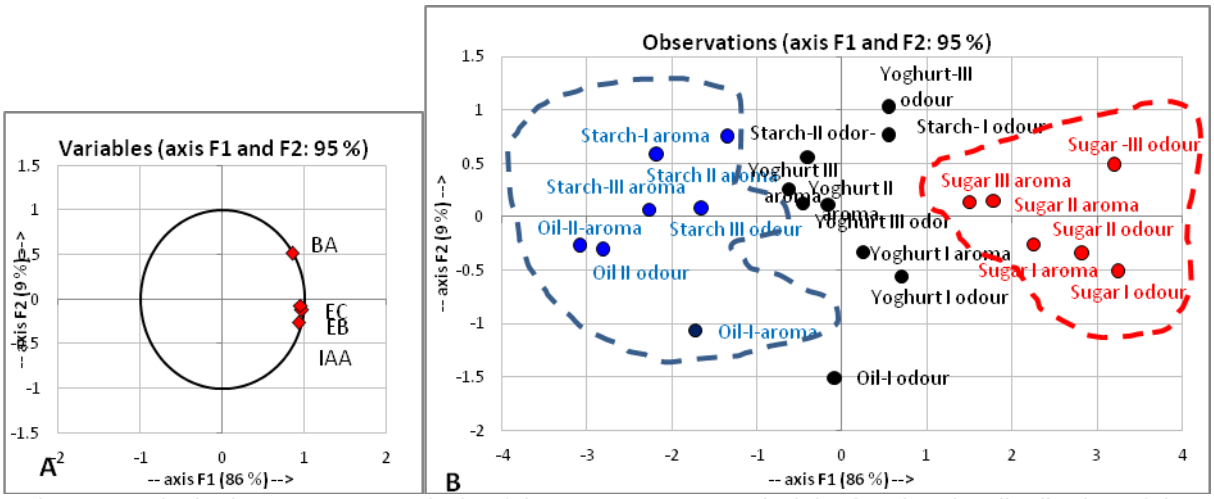

Figure 5. Principal component analysis of the aroma compounds (A), showing the distribution of the samples: I, II, III means three levels of viscosity; (B)

All quantitative data of sensory evaluation of investigated aroma compounds were subjected to PCA analysis in order to find correlations among aroma compounds (loading plots Fig. 5.A). 86% of the total variance was explained by the PC1 and all aroma compounds contribute to the PC1. The sample sites located on the score plot (Fig. 5-B) show clearly the distribution on two clusters. The first one is on the right side of the plot and represent sugar food matrix and all 3 levels of viscosity which contribute to enhancing, specially, odour but also aroma intensity for all aroma compounds. On opposite side located starch and oil matrix which decreased aroma intensity for any level of viscosity. From the plot it could be seen that aroma intensity of all aroma compounds is perceived lower then odour intensity. Several authors (Voirol and Daget, 1986; Pierces and Halper, 1996) who investigated differences between orthonasal and retronasal olfaction concluded that those differences could be explained as a less efficient access of volatiles compounds to the sensory area when they follow the retronasal way.

CONCLUSIONS

It was found that the viscosity of sugar, starch and oil matrixes influence on aroma release while the viscosity of yogurt did not significantly influence on delivering aroma to human sensory receptor. Sucrose enhanced perception of aroma compounds and yoghurt enhanced aroma intensity of benzaldehyde. Transmission of aroma compounds to the oral cavity and olfactory epithelia from oil, starch paste and yoghurt matrixes were hindered. Perceived aroma intensity (retronasal) was less then perceived odour intensity (orthonasal) for all tested aroma compounds.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project “Olfaktorno utvrđivanje intenziteta oslobođenih aromatskih jedinjenja iz različitih matriksa hrane“ (Olfactory Evaluations of Intensity Realised Aroma Compounds from Different Food Matrixes) financed by the Federal Ministry of Education and Science, Bosnia and Herzegovina.

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ATTITUDE OF THE CONSUMERS DIFFERENT AGES AND EDUCATION LEVEL TOWARD FUNCTIONAL PRODUCTS BASED ON CEREALS

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ABSTRACT: The aim of this study was to quantify consumers attitude towards functional products based on cereals and to find the underlying dimensions that can predict respondents' willingness to use these products. For the analysis of consumers attitude were used a multivariate methods MANOVA and discriminant analysis. Roy's-test, Pearson coefficient of contingency (χ), multiple correlation coefficient (R) also applied. When the results in relation to the education level of respondents are observed, it was found that highest difference among respondents was in their attitude regarding health status information whereas the least difference was in their attitude regarding the way of presenting information about functional product based on cereals. The introduction of functional products based on cereals will not automatically be successful without the simultaneous introduction of information that is of use to consumers in making informed choices about purchase and inclusion in their diets.
Key words: *consumers, functional foods, cereals*

INTRODUCTION

Together with the development of consumer awareness and changes their behaviour, the needs for quality products with precisely defined nutritive and other characteristics are increasing. In the last decades consumer demands in the field of food production has changed considerably. Consumers more and more believe that foods contribute directly to their health (Mollet, Rowland, 2002). Consumer benefits from the consumption of so-called "functional" foods are potentially very wide-ranging, and are associated with different advantages to human health and quality of life. The term "functional food" has been linked with various definitions that vary from simple statements to rather more complex ones. Consumer acceptance of the concept of functionalfoods, and a better understanding of its determinants, are widely recognized as key success factors for market orientation, consumer-led product development, and successfully negotiating market opportunities (Ares, Gámbaro, 2007). The assumption that functional products with specific health advantages are likely to deliver population-wide benefits is not now generally accepted.In recent years, cereals have also been investigated regarding their potential use in developing functional foods. Cereals are grown over 73% of the total world harvested area and contribute over 60% of the world food production providing dietary fibre, proteins, energy, minerals, and vitamins required for human health. Most research on functional foods is concentrated to possible health effects, We do not know too much about consumer response to these products (Saher et al., 2004). The addition of vitamins and minerals in cereals, known as food enrichment or fortification, is a common practise in developed countries used to prevent vitamin and mineral deficiency in the diet. The main components used in white flour fortification are thiamine, riboflavin, niacin, folates and iron. The fortification of cereals was technologically available from the point when it was possible to synthesize pure forms of vitamins and minerals which were controllable. It should be noted that food fortification made it possible to find the causative relation between nutrient deficiency and occurrence of degenerative diseases. The main argument that contributes to better sale of functional food is the claim that it is beneficial for health which particularly affects younger generations

seeking for higher quality of life (Cross, Frost, 2009). Increased demands after healthier and more nutritious products is a consequence of more conscious consumers which contributes to constant necessity for new products and increased differentiation of product assortment (Lineman 1999., Deliza et al., 2003).Consumers have been increasingly interested in health effects of food or their components. As an answer to these demands, food industry makes efforts to provide various food products enriched with functional components (Position of the American Dietetic Association: Functional Foods 2009).
Previous studies have shown that:

- Consumers are sceptic towards nutritive and health claims,
- Consumers are not able to clearly distinguish between these claims (although they perceive them as useful),
- Consumers prefer shorter claims rather than complex ones (Verbeke et. al. 2009).

These statements are probably the consequence of lack of motivation and knowledge on basic physiological functions. It is obvious that the particular health benefit is more useful for the consumer than the way it is presented (Van Trijp, Van der Lans, 2007).
However, consumers rarely prefer or dislike a food on the basis of business orscientific terms describing its healthiness or nutritional value (Lähteenmäki, 2003).

MATHERIAL AND METHODS

The research was conducted through randomly distributed survey on the teritory of Novi Sad. The consumers attitude has been tested on a sample with 508 respondents divided to subsamples based on: ages, education and gender.
Ages:

- <18 year-140 respondents,
- 18-30 year-67 respondents,
- 31-50 year-237 respondents,
- >50 year-64 respondents.

Education:

- Primary school-103 respondents,
- High school-338 respondents,
- Faculty-67 respondents.

Gender

- Male
- Female

For the analysis of consumers attitude a multivariate methods MANOVA and discriminant analysis were used. Roy's-test, Pearson coefficient of contingency (χ), multiple correlation coefficient (R) were also applied. The results are further analyzed by estimating the homogenity of subsamples, distance among them and Cluster analysis (Anderson, 1984).

RESULTS AND DISCUSSION

The entire study was conducted in thematic groups, and analyzes the difference between: age, education and gender of respondents in relation with the following units:

- Consumers attitude about their health ,
- Consumers attitude about understanding nutritional and health claim,
- Consumers attitude about purchase of functional products based on cereals,
- Consumers attitude way of information about functional products based on cereals

The higher the discrimination i.e. difference among the subsamples, the more expressive are subsample characteristics. The percent of contribution (%) for each entirety shows how the particular whole defines the characteristics of a subsample in relation to other entirety. It is also given an overview of characteristics with homogeneities of each subsample in relation to the measure of discrimination and degree of ascendingly sorted characteristics. The

contribution of entirety (space) to characteristics and contribution of characterisctics within space are important parameters that define the hierarchy within the spaces and order of the characteristics.

Table 1. The contribution of the entirety to characteristics

Contribution %	Consumers attitude	subsamples
12.088	about their health	education level
12.035	about their health	ages
11.476	way of information	ages
11.094	understanding nutritional and health claim	education level
10.509	purchase of functional products	education level
10.331	understanding nutritional and health claim	ages
9.765	purchase of functional products	ages
7.929	way of information	education level
4.238	purchase of functional products	gender
4.139	about their health	gender
3.738	way of information	gender
2.658	understanding nutritional and health claim	gender

The contribution of total to characteristics of subsamples (%) (Table 1.), showed that the highest difference between respondents was in their statements regarding health information in relation to their education level and age. There were also significant differences between the respondent statements regarding the way of presenting information about functional food in relation to the age group of respondents as well as their statements regarding understanding nutritional and health claims in relation to education level and age of respondents. On the other hand, least differences were observed with respondent attitudes about buying functional food, information on health status, way of presenting information about functional food, understanding claims in relation to the gender of respondents.

Table 2. The contribution of the entirety to characteristics

Contribution %	Consumers attitude	subsamples
28.686	purchase of functional products	gender
28.018	about their health	gender
25.301	way of information	gender
17.996	understanding nutritional and health claim	gender

Analysing the results in relation to the gender of respondents (Table 2.), it was observed that highest difference among respondents was in their attitude regarding buying functional foods whereas the least difference was in their attitude about understanding claims.

Table 3. The contribution of the entirety to characteristics

Contribution %	Consumers attitude	subsamples
27.599	about their health	ages
26.317	way of information	ages
23.691	understanding nutritional and health claim	ages
22.393	purchase of functional products	ages

Analysing the results in relation to the age of respondents (Table 3.) it was observed that highest difference among respondents was in their attitude regarding health status information whereas the least difference was in their attitude about functional product purchase.

Table 4. The contribution of the entirety to characteristics

Contribution %	Consumers attitude	subsamples
29.043	about their health	education level
26.656	understanding nutritional and health claim	education level
25.249	purchase of functional products	education level
19.051	way of information	education level

When the results in relation to the education level of respondents are observed (Table 4.), it was found that highest difference among respondents was in their attitude regarding health status information whereas the least difference was in their attitude regarding the way of presenting information about functional product based on cereals.
There was a clearly distinguished difference among certain education levels of respondents with regards their statements on health status information.

CONCLUSION

The introduction of functional products based on cereals will not automatically be successful without the simultaneous introduction of information that is of use to consumers in making informed choices about purchase and inclusion in their diets. The approach being most widely adopted to inform consumers is that of market segmentation, with origins in market research. Understanding consumers' risk perceptions and concerns associated with processing technologies, emerging scientific innovations and their own health status may enable the development of information strategies that are relevant to wider groups of individuals in the population, and deliver real health benefits to people increasingly suffering from illnesses relating to ageing and over-nutrition. Research has shown that ages and education level can significantly affect to consumers' understanding of nutritional and health claims: The most important factors are: the availability of information, age and education level.

- With increasing consumers age also increasing interest of consumers to read the content on the nutritional and health claim.
- Older consumers better understand information presented on nutritional and helath claim.
- Age of consumer significantly affected their attitude as regard to which information on the claim is the most important.
- Older consumers usually read the labels and possess enough knowledge on the elements of labelling.
- Consumer age affects the consumer's reliance over health claims. More educated consumers consider information presented in nutritional and health claim important for proper choice of product and perceive it as an inherent part of a quality product.
- Consumers with higher level of education know more about what nutritional and health claim should contain.
- More educated consumers more frequently check the title of producer on the label and the nutritional and health claim.

Based on the influences of:gender, age and education level to consumers attitude toward information provided by the nutritional and health claim, it is possible to predict the requirements and models of their behavior.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from the Ministry of Education and Science of the Republic of Serbia (Project III 46005).

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IMPROVEMENT OF PRODUCTION AND PLACEMENT OF TRADITIONAL DAIRY PRODUCTS IN SERBIA

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ABSTRACT: Traditional food products represent a heritage and are the result of accumulated empirical knowledge that is passed through the generations.
Dairy products, including cheeses and kajmak, have a long tradition of production and consumption in Serbia. The two known groups of autochthonous Serbian cheeses are white brined and pasta filata cheeses which are represented in central and south parts of Serbia, respectively. Kajmak is a dairy product with unique composition and sensory properties and with great potential to be recognizable brand of domestic agriculture.
Nowadays, the most of these products are usually produced in households and small craft dairy plants which are poorly organized. Such manufacturing methods of traditional dairy products differ greatly from region to region, yielding products of uneven quality and safety with wide variation in composition and characteristics. All of these are certainly the main reasons why such a delicious dairy products are not recognized as traditional products with protected origin as well as have been left out of the world market and remains only significant on a local level.
This paper discusses the main problems and difficulties of the traditional dairy products manufacture such as organization of primary milk production, quality of raw milk, conditions in the production dairy plants, lack of knowledge etc.
Also, the available and missing resources in traditional dairy products manufacture were considered in order to indicate the possibilities and the main ways for improvement of traditional production and placement on market. The main goals in future should be based on the detailed defining of composition, properties and method of production of selected dairy products, establish better control, better connection between different public and private Institutions, associations and collective actions of producers on market, the education of producers as well as consumers etc.
The increasing of the supply of products with tradition and geographical designation of origin should be an important commitment for rural development as well as overall agriculture in Serbia. All of these can help for better positioning and distribution of valued traditional Serbian dairy products on domestic and world markets.
Key words: *traditional production, cheese, kajmak,*

INTRODUCTION

Traditional food represents a cultural heritage and is the result of accumulated empirical knowledge passed from generation to generation. Production and consumption of different traditional food, including dairy products, has a long tradition in Serbia (Dozet et al., 1996b, Dozet et al., 2006). All types of traditional products differ with unique composition and properties and are associated to the territory of their origin included the production methods, climatic conditions, habits, environmental conditions, etc. However, nowadays, these products are still significant just on a local market and have been left out of the world market.
This article is focused on the short review of the main characteristics of autochthonous Serbian dairy products, including two most popular groups of cheeses and kajmak, as well as present difficulties in the traditional production of these products. Also, considering the resources in the traditional production, possible directions for its improvement and their better positioning on market are discussed.

Traditional Serbian dairy products

The most famous dairy products in Serbia whose production dates back to the past are cheeses and kajmak. Within the group of cheeses the best known are two following types: white brined and pasta filata cheeses. The main characteristics of these dairy products as well as basic steps of production procedure were given below.

White brined cheeses

White Brined cheeses (WBC) are the most important family of cheeses for Serbia and neighboring countries (Mediterranean region). The cheeses of this family are the most widely produced and consumed cheeses in Serbia and represent about 60 % of total cheese consumption (Dozet et al. 2006). There are many different types of Serbian brined cheeses, named according to their production regions as follows: Sjenica, Zlatar, Svrljig, Homolje cheese (Dozet et al., 1996a). These cheeses may be very similar, but they also rather different in respect of the milk type, region of production, manufacturing protocols, composition and sensory properties, etc. Traditionally, they were usually made from cow's, sheep's and goat's milk (Dozet et al., 1996b), but nowadays they are mainly produced from cow's milk. However, all of them have the characteristic of being ripened and stored in brine until their consumption.

Currently, WBC in Serbia are produced by both traditional and industrial methods. Traditionally, white brined cheeses have usually been made from unpasteurized or medium heat-treated milk, without starter cultures and in small dairy plants and households using simple equipment (Dozet et al., 1996a,b). The general procedure of WBC production involve next steps: raw milk heating, addition of $CaCl_2$ and starters at 30– 35^0C if they are used, renneting and coagulation (40-60min.), cutting into 1–3-cm cubes, rest for 10–15 min, moulding into rectangular or cylindrical moulds, draining (usually without or under low pressure), cutting the curd into the final cheese dimensions, salting (in brine or with dry salt), packaging in containers and ripening at 16–18^0C (until pH reaches a value of 4.6) and storage at 4-8^0C. Because of the low pH, the high salt content and the relatively short ripening period, biochemical changes are not extensive during ripening.

The colour of WBC ranges from off-white to yellowish when they are made from cow's milk. They have no rind and gas holes, but the other openings should be present in the cheese mass. The texture of WBC is smooth, soft and crumbly but still sliceable. Their shape varies and depends on the package container. Usually cheese blocks are rectangular or cubic and weigh between 250–1000 g.

Pasta filata cheeses (Kashkaval)

Pasta filata (PF) cheeses are the second most popular cheese group in Serbia and East-Mediterranean countries (Dozet et al., 1996a, Pudja and Milanovic, 2000., Alichanidis et al., 2008). Production and consumption of cheese is associated with the southern part of Serbia, around the city Pirot, and the most famous cheese in this group is Pirotski Kashkaval (Pudja and Milanovic, 2000). Currently, the most of pasta filata cheeses are produced on industrial scale using the modern equipment. However, traditional production of Kashkaval is still present in some rural areas in Serbia.

Traditionally, they are produced from raw sheep's milk but today the most cow milk is used. The manufacture of Kashkaval cheese consists of two independent stages: (i) production of the curd and its acidification (cheddaring) (ii) texturizing of the acidified curd (heating, kneading and stretching in hot water or brine). General production procedure consists of following phases: heat treatment of raw milk until coagulation temperature (31-34^0C), coagulation with rennet, cutting of curd (6–8-mm cubes), left for about 10 min then scalded to 42^0C for 35 min with stirring, collecting of curd mass and forming of cheese blocks. Then, curd blocks left to be acidified (cheddaring process) until pH reaches a value of 5.2 which is the most important step in pasta filata cheese production. Curd acidification to a certain extent is of importance since it results in the characteristic fibre-like structure of the final cheese. The acidified curd is cut into long thin slices and texturised by soaking the cheese mass in a hot water or brine (80-85^0C) using the modern equipment or with intensive manual

labour in industrial and traditional method, respectively. Traditionally, the curd mass is placed into wooden baskets, immersed in hot water and manipulated with a wooden stick until a homogenous compact structure is obtained. The hot curd was then transferred to a table and hand-kneaded like dough, partially salted and moulded in metal or wooden hoops. A small amount of salt is applied 4-5 times to the cheese during the first 2-3 weeks of ripening. Cheese are left to ripen for about 2–3 months 12–15^{0}C/RH 85%) and storage 2–4^{0}C (Pudja and Milanovic, 2000., Kindstedt et al., 2008).
The typical form of Kashkaval cheese is flat, cylindrical, with a smooth, amber-coloured rind. The texture of all Kashkaval-type cheeses is laminar, elastic, very close with visible layers, occasionally with random slots but without gas holes. The National Committee for Standardization in Serbia has defined the National Standard for Kashkaval cheese (Standard SRPS E.C2.010:1997). The Standard takes into account traditional manufacturing practices, as well as modern trends in the industrial production of the cheese. According to the Standard, 'Kashkaval is a semi-hard or hard, pastafilata cheese which is available in two types: Kashkaval (weigh 5-10 kg, diameter 30 cm and 10-13 cm in height) min. 56% dry matter, min. 45% fat in dry matter and min. 8 weeks ripening period) and Kashkaval Krstas (weigh up to 3 kg, min. 54% dry matter, min. 45% fat in dry matter and min. 4 weeks ripening period). Both cheese types can be produced from cows', sheep's, goats' or mixed milk, which may be raw or pasteurized.

Kajmak
Kajmak is a dairy product with unique composition and sensory characteristics. Nowadays, kajmak is usually produced in households and small dairy craft plants by traditional method. In the past, the several attempts have been proposed as a solution for industrializing of kajmak production but unfortunately there still is no significant application of them (Pudja et al., 2008).
Traditional kajmak procedure is based on the surface activity of the top layer of boiled milk. Hot milk is poured into the open shallow vessels where, due to surface activity and evaporation, a kind of initial skin is formed on the top of the milk. After initial skin formation, a gradual process of milk cooling takes place and lasts about 24 h, with a final temperature that ranges from 10 to 15^{0}C. Important factors that control the process of kajmak formation are: milk composition, milk temperature, the humidity and temperature of the surrounding air, and the temperature difference between the milk and air. The kajmak formed on the top of the milk is collected, salted and, layer by layer, placed in appropriate vessels. It may be consumed immediately after manufacture, as a fresh kajmak, or after a maturation period, as a ripened kajmak. Maturation of kajmak usually takes place 15–18^{0}C at least over 15–20 days, but also can be stored in cold conditions (below 8^{0}C) over 3–6 months, and in some cases up to one year (Pudja et al., 2008).
The color of kajmak is mostly influenced by the ripening time, composition of milk fat and milk type. Fresh kajmak has a light color ranging from white to ivory, while ripened product differ with light to dark yellow. The flavor of fresh kajmak is mild, creamy and milky which become more intensive and stronger due to numerous volatile, aromatic compounds formed from proteolytic and lipolytic processes undergone during ripening. The consistency and structure of fresh and mature kajmak vary widely. Fresh kajmak is characterized by a soft, creamy consistency and good spreadability. Mature kajmak has a harder and grainy structure due to its higher total solids level, but also more spreadable structure (Pudja et al., 2008).

Designation of origin and geographical indication
In last 20 years, special attention was given on the protection of origin of different food, including dairy products, which differ with long tradition of production within some specific regions. Traditional Serbian dairy products which are described above are still not recognized as specific products with signs of geographical origin. However, Homolje cheeses, which belong to WBC, are protected with sign of protection geographical origin on a local level and according to domestic Regulations (Radovanovic et al., 1996), but they are still out and without significant recognition on wide dairy products market.

The idea of protecting and preserving diversity of traditional foods, including cheeses, is defined by the Paris Convention back in 1883rd year. The term "*Appelation d'Origine Contrôlée*" (AOC) was first time introduced in this document which identified the value of food in specific region and clearly defined quality and authenticity of such products. In Europe, this concept was widely accepted 1992nd (Regulation EC 2081/92) and current Council Regulation (EC) No 510/2006 is valid. This Regulation defines all terms and different levels of food origin protection. EU Regulation sets out provisions three types of geographical description on agricultural products and foodstuffs from a defined geographical area. A PDO (Protected Designation of Origin) covers the term used to describe foodstuffs which are produced, processed and prepared in a given geographical area using recognized know-how (such as Mozzarella di Bufala Campana, Italy). A PGI indicates a link with the area in at least one of the stages of production, processing or preparation (such as Canestrato di Moliterano, Italy). The link with the area is therefore stronger for PDOs. Traditional speciality guaranteed (TSG) sign emphasizes a product's traditional composition or traditional mode of production (Boerenkaas, Netherland; Mozzarella, Italy) (www.eu.europa.eu).

Currently, about 1320 products (excluding wines and spirits but including beer) were defined with PDO, PGI and TSG marks in the European Register. The main countries in terms of value of PDO/PGI production are Italy (33% of the total), Germany (25%), France (17%) and the United Kingdom (8%). In addition, it is estimated that part of the production of 30% of protected products are exported outside the EU. Cheeses account for third of total PDO/PGI products (~35%) and represent ~8% of total cheese produced in EU (3-4% of world production). The study carried out by the European Commission's shows a constantly rise in exports of these products in last few years (www.eu.europa.eu).

The use of corresponding EU symbols on the labels of specific food products encourage the diversification of agricultural production, protect the product names from misuse and imitation and provides consumers with clear and concise information on their origin. The introduction of these terms also benefits the rural economy, since it boosts farmers' income and maintains the population in less favoured or remote areas.

As it was said above, the good example of improvement production and placement of traditional foods, regarding to cheeses, are Italy, Switzerland and France which differ with very well organized and developed agriculture sector.

In Italy 1 millions of tons of cheeses are produced and more than 450.000 tons are cheeses with designation of origin (www.clal.it). The most of cheeses are consumed on domestic market, but also it is very important export of them which contribute significantly to country economy. Namely, Italy exports almost 270.000 tons of cheese, with a value of 1.4 billions of euro (www.wds2011.com). The export of protected cheeses is within 10 to 28% of total cheese production, with constant rise, depending on the cheese type. Main Italian exported cheeses are Mozzarella and other fresh cheeses (~36%), Grana Padano PDO and Parmigiano Reggiano PDO (~25%), Pecorino Romano PDO, Gorgonzola PDO and Provolone (www.clal.it, www.wds2011.com). Similar data can be analyzed for other countries such as France, Swiss and for the last few years Spain, Portugal etc.

However, it is evident that protection of traditional production of food may have positive influence on overall economy as well as for rural development of country.

The problems and recommendation for improvement of production and placement of traditional dairy products

Traditional production of food, including dairy products, in Serbia is faced with numerous problems and difficulties which result in non adequate positioning of such products on the domestic and world market.

Traditional production of dairy products is strong connected with changes of primary milk production as well as livestock in some regions. Namely, the great migration of population from rural areas has contributed to closing of numerous farms. Currently, in Serbia the production of raw milk (about 65%) is mostly connected with high numbers of small farms, with 1 to 5 cows (Popovic, 2008). This is extremely small if compared with EU-15 where the average herds size is around 40 cows, ranging from 14 cows per holding in Greece to 80 in

the UK (Berkum, 2007). High number of farms, small scale production and shortage of awareness for cooperative action makes this ring in whole dairy chain the weakest. Because of that one of the main measures of agricultural policy should be aimed to education and stimulation of dairy farmers through Extension service to organize cooperatives.

The poorly organized primary production results often in bad quality of raw milk which has a direct impact on dairy products quality and safety. As mentioned above, the most of traditional dairy products are produced in housholds and small scale dairy plants. Currently, about 40% of raw milk market is controlled by 16 middle sized and 180 small sized dairy companies but the actual data about milk amount which is processed in households is still unknown (Popovic, 2008). The often bad hygienic conditions in these type of plants and housholds contribute to substanial safety risks and poor microbiological quality of traditional dairy products. Moreover, the procedure of dairy products manufacture differ greatly from region to region, yielding products of uneven quality with wide variation in composition and characteristics which also prevents the proper positioning of them on the market. Also, it is important to emphasize that until now and regarding to protection of geogrpahical origin the manufacturing protocols of the most autochthonous dairy products are not well defined. Hence, one of the most priority in future is detailed defining of raw milk quality, composition and properties as well as production protocols for typical Serbian dairy products. On that way it is possible to start the serious procedure for the geographical protection of orgin on the local as well as on the EU level.

Traditional dairy products, such as cheeses and kajmak, are usually produced from raw milk without using starter cultures. Specific native microflora has an inportant role in the numerous events during ripening of traditionally made products and forming their specific sensory properties. Hence, due to poor manufacturing conditions, there is uncontrolled growth and activity of endogenous microflora during production and ripening processes, leading to potential safety risks and spoilage of final products. On the other hand, the use of commercial starter cultures as well as introduce of heat treatment, which is common for industrial production, enables improvement of safety aspects, but impairs cheeses specific sensory properies. The autochthonous microflora is claimed to play a major role in determination of cheese specificity. Biodiversity of cheese microflora is very significant because their selection enable obtaining authentic starter cultures for standardization and protection designation of origin for these traditional cheese types. The diversity of microflora within the area of origin has been the subject of study in several Serbian traditional cheeses (Radulovic, 2010, Martinovic et al., 2008). For the improvement of the authentic microflora issue, it is necessary to isolate lactic acid bacteria (LAB) from traditional cheeses and evaluate their technological properties, in order to test their potential application as components of defined starter cultures (Radulovic et al, 2011).

Application of selected microorganisms may help to improve a product safety with remaining the unique and specific sensory properties of autochthonous dairy products. Such kind of traditional cheese standardization is known for well organized country as Switzerland. Cheeses, such as Emmental, Gruyer etc., with PDO sign, are usually produced in numerous small scale family plants from raw milk, sometimes with addition of LAB selected from traditional made cheeses (Saric et al., 2007). However, it is important to emphasize that the main role in selection of LAB, quality control and research activities as welll as supplying producers with unique starters, belong to the biggest scientific Institute. Based on that, it is clear how is important to make strong connections between dairy products producers and scientific institutions. In Serbia, there are several scientific institutions working on autochthonous LAB research, but with the insufficient level of collaboration. One of the priorities should be merging the collections of LAB, isolated from particular cheeses in order to establish the base for supporting and upgrading the production of the autochthonous dairy products. This may also be a meaningful step in efforts to establish the integral concept for the certificates of origin issue for various dairy products.

The specific production procedure of some traditional products such as kajmak also may present the obsticle for better organization and getting the high quality product. Namely, traditionally kajmak production includes numerous steps that represent potential safety risks

for kajmak and the remaining milk such as: (i) the vast surface of the milk layer in contact with the surrounding air; (ii) the long-lasting process of milk cooling with a very long period at a moderate temperature able to stimulate microbiological growth and (iii) the low level of technical support and the transfer of the different products by hand (Pudja et al., 2008). Regarding this, it is necessary to find a solultion for improvement and modifications of some steps in kajmak production which will enable to get product with high quality and safety. The improvement of kajmak production should be based on the shortening of the production time, introducing the constant and defined environmental conditions, the use of selected starters, better processing control, but with keep all characteristics that are typical for traditionally-produced kajmak. In that activities also the scientific institutions may participate significantly.

An important characteristic of traditional production, as well as the majority of the primary production and/or processing, is lack of knowledge and cooperative actions (beetween Institutions and producers). In practice, there are just few associations of dairy farmers and dairy products producers, that don't have any economical or political influence on market or related institutions. In developed countries such are Italy, France and Switzerland the existing of that organisation is key factor for common/joint participation and succes on market. Therefore, it is necessary to establish a better relationship between all relevant actors in the chain, such are scientific institutions, local authorities and traditional products manufacturers. For overcoming this problem, the formation of association on regional and national level is of primary importance, as well as inclusion in international and world associations (such as World wide Traditional Cheese association, established and based in January 2012. in Ragusa, Italy) is very helpful for gaining and exchanging experience.

The absence or malfunctioning of the control and certification procedures is a major problem for all types of quality signs and damages the credibility and further development of the protected products and quality sign labels. In order to establish an effective control system can be recommended actions include technical assistance projects involving an international expertise are required, training and study tours.

The next request which may help to getting quality sign and recognizable lables of Serbian traditional products is harmonization of the existing regulations with EU regulation model. What is more into question is the way the regulations are understood and implemented by the institutional stakeholders.

Institutional networks involved in the protection of geographical indications (GI) of traditional foodstuffs should be enlarged. Currently, so far the responsibilities related to the protection and promotion of GI are mainly concentrated on the Offices for Intellectual Property which leads to an overestimation of the legal aspects and to a poor consideration for practical agricultural and rural development issues, as well as for market issues. The role of the national and local agricultural institutions, local authorities, economic institutions and agencies and research institutes needs to be better defined or re-defined. Increasing awareness of producers/processors on the opportunities which could be offered by the PDO/PGI system is also important and can be achieved through organization of study tours end exchanges with farmers and producers in developed countries (EU, Switzerland), publication of information material and guidelines for farmers, producers and local authorities. However, it can hardly be achieved on a proper way if the legal and institutional framework and the objectives are not clearly defined.

At the end, increasing the visibility of quality sign products on the market and awareness of the consumers is very important from economic impact of any geographical indication protection. Hence, the awareness of the consumers should be achieved through market and consumer surveys, trainings, specialized tours, campaigns, adoption of logos and labeling for some local markets.

CONCLUSION

Serbia has a long tradition of production and consumption of dairy products, especially white brined and pasta filata cheeses and kajmak. However, these products, besides their unique and specific properties, have been left out of the world market and remain significant only on

a local level, due to poor organized and uncontrolled primary production and processing of raw milk, lack of knowledge and cooperation actions between different Institutions and producers etc.
Establishment of conditions for getting sign of protection origin (PDO, PGI, TSG) represents national interest in field of agriculture and rural development in Serbia. As primary assignment, it is necessary to make better organization and connections between all important segments and phases during production of autochthonous dairy products. Improvement of production should be based on the detailed defining manufacturing methods, composition and properties of selected products, establishing better control of all phases during production.
This complex problem requires engagement all national responsible authorities, through promoting, planning and coordinating activity of harmonization of regulations, cooperation actions, education of producers, and sensitizing public awareness on the importance of protecting and valorizing traditional dairy products and developing marketing strategies.
Better positioning of traditional dairy products in Serbia represents an important potential in rural development and domestic agriculture, having a direct effect on development of trade and placement of our traditional dairy products in domestic and foreign markets.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III 46009 financed by the Ministry of Education and Science, Republic of Serbia.

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THE RIPENING OF WHITE BRINED CHEESES MADE WITH COMMERCIAL AND POTENTIAL AUTOCHTHONOUS PROBIOTIC STRAINS

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ABSTRACT: The white brined cheeses are the most popular cheeses with the long tradition of production and consumption in Serbia. Modern medical research studies indicate the importance of diet in the maintenance and improvement of health. In this regard, for the last decades, there is rapid growth of food products with dietetic and functional properties, including products with probiotic bacteria, which could be beneficial for human health. Milk products, including cheeses, represent a good base for the development of new products with functional properties, especially those with probiotic bacteria.
The effects of commercial and autochthonous strains of lactic acid bacteria on the composition and proteolysis of white brined cheeses were studied throughout 30 days of ripening. Cheese A was produced with commercial strains (LL 50 A and MY 721, DSM, Netherlands) *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris* and probiotics *Lactobacillus acidophilus* and *Bifidobacterium lactis* and cheese B with autochthonous strains *Lactococcus lactis* ssp. *lactis* 563*, Lactococcus lactis* ssp. *cremoris* 565 and potential probiotics *Lactobacillus plantarum* 564. The rate of proteolysis was analyzed by the water and 5% phosphotungstic acid soluble nitrogen fractions, as well as by SDS and UREA PAG electrophoresis. The viability of probiotic strains during cheeses ripening was also determined.
A significant influence of different starter and adjuncts bacteria on the composition was not found. The proteolysis rate was significantly different ($p<0.05$) between cheeses made with different starter cultures, due to different proteolytic activity of bacteria used. The viability of adjunct probiotic bacteria in both cheeses were maintained on the high level during the overall ripening period that are necessary for acquirement of their therapeutic effects.
Cheeses made with autochthonous bacteria showed a higher rate of secondary proteolysis, as well as higher flavour scores, and were more acceptable than cheese made with commercial probiotic bacteria.
Key words: white brined cheeses, lactic acid bacteria, probiotics, proteolysis, sensory properties

INTRODUCTION

Probiotics are “living micro-organisms, which upon ingestion in certain numbers (10^7 cfu/g) exert health benefits beyond inherent basic nutrition”. Foods containing such bacteria fall within the “functional food” category and the market for such products has rapidly grown for last twenty years (Saarela et al., 2000).
Milk products, including cheeses, represent a good base for the development of new products with functional properties, especially those with probiotic bacteria (Ross et al., 2002). The white brined cheeses are the most popular cheeses with the long tradition of production and consumption in Serbia (Dozet et al., 2006). The most of them are still produced in small scale production plant and often are characterized with various composition and properties. However, there is limited knowledge about white brined cheese produced with probiotics. Currently, many different strains and species of lactobacilli and bifidobacteria have been used commercially as probiotics (Ong et al., 2006, Bergamini et al., 2006). However, for last decades numerous researches carry out in direction to find new probiotic strains (KiLiç et al., 2008). Considering the fast growing interest for application of

probiotic strains in dairy products, it could be presumed that it is possible to isolate some strains, from different food, including traditional made cheeses, with potential probiotic ability, among the autochthonous strains (Radulović, 2007, Radulović et al., 2010a, b).
The objective of the present study was to analyse influence of commercial and potential autochthonous probiotic strains isolated and selected from traditional made cheeses on the composition and proteolysis of white brined cheeses.

MATERIAL AND METHODS

Cheese manufacture

The white brined cheeses were made from fresh cow`s milk which was heated at 72^0C/30s, then cooled until 34^0C and 200 mg/l $CaCl_2$ was added. Two different starter cultures were used as follows: cheese A was produced with commercial lactococci (LL50A, DSM, Netherlands) and probiotics *Lactobacillus acidophilus* LAFTI®L10 and *Bifidobacterium lactis* (MY 721, DSM, Netherlands) and cheese B was produced with autochthonous lactic acid bacteria which are consisted of strain *Lactococcus lactis* ssp*. lactis* 563 ($1.7x10^{11}$/ ml),), *Lactococcus lacti*s ssp. *lactis* 565 ($2.5x10^9$/ml) and potential probiotic *Lactobacillus plantarum* 564 ($1.8x10^9$/ml) in ratio 1:1:1 (Collection of Microbiology Laboratory, Faculty of Agriculture, University of Belgrade, Serbia). Then calf rennet (Sacco Clarici, Italiy) was added and the coagulation took place in 45 min. at 32^0C. Once curdling was completed, the coagulum was cut into small pieces (2–5 cm) and stirred three times for 5 min. during 30 min. The cheese mass was carefully transferred from cheese vats into the mold with cheese clothes. After about 1h of draining (without pressing), the pressure was applied (max. 3kg/kg) for 3h. Then, the cheese curds were cut into pieces of 10x10x7cm with knife. The curd blocks were dry salted with 2.4% NaCl. The ripening took place in brine (8%) at 12^0C during 30 days. The cheeses were sampled and analyzed after 1, 7, 15 and 30 days of ripening.

Analytical methods

Cheese samples were analyzed in duplicate for dry matter (DM), fat (MF), total protein (TP) and salt content (NaCl) by standard methods (Carić et al., 1998). The pH of cheese slurry was measured by a pH meter (Consort, Belgium).
Proteolysis was also studied by means of water-soluble nitrogen (WSN) according to the method of (Kuchroo and Fox, 1982), and phosphotungstic acid (5%) soluble nitrogen (PTAN), according to (Stadhousers, 1960), both expressed as a percentage of the total nitrogen matter (WSN/TN and PTAN/TN).
SDS PAG electrophoresis of cheese samples was performed according to Laemmli (1970) method, using a vertical slab unit TV200YK (Consort, Belgium) with 100x200x1mm slabs, Tris-glycine electrode buffer, constant current of 80 mA, a max. voltage of 300V for 4 h, with 4% stacking gel (pH 6.8), and 12% separating gel (pH 8.9). Urea Page was conducted according to Andrews method (1983) using the same equipment.
Data were analysed using STATISTICA 6.0 (StatSoft, USA) data analysis software. LSD test was used to determine differences among cheeses at a 0.05 statistical level.

RESULTS AND DISCUSSION

The composition of the various cheeses during ripening is summarized in Table 1.
The brined cheeses, according to fat in dry matter and moisture in fat free content, belong to full fat and soft cheese category (Table 1). Our results are in agreement with literature data about white brined cheese composition (Macej et al., 2006). Generally, white brined cheeses showed significant variations of composition due to different production method. There were no significant differences ($P>0.05$) in the gross composition of brined cheese made with commercial and autochthonous lactic acid bacteria (Table 1.). Similar studies were reported in previous studied (Ong et al., 2006, KıLıç et al., 2008). Most of the cheese composition parameters did not change significantly ($P>0.05$) during 4 weeks of ripening. However, a

significant increase of dry matter content (P<0.05) after 7 days of ripening was detected, probably due to salt absorption in cheese, as well as water diffusion from cheese into brine. pH values of cheeses were regularly decreased (data not shown) indicated on the good acidogenic ability of autochthonous lactic acid bacteria what is especially important for their use as starter culture in cheese production. Also, the numbers of starter and adjunct probiotic bacteria were maintained on the high level during all investigated time (data not shown). This is especially important for acquirement of probiotic bacteria therapeutic effects (Ross et al., 2002).

Table 1. Composition of white brined cheeses produced with commercial and autochthonous starter cultures during 30 days of ripening

Day of ripening	Cheese	Dry matter (%)	Total Protein (%)	Fat in Dry matter (%)	Moisture in free fat basis (%)
1	A	39.43±0.15a	13.57±0.60a	51.36±1.81a	75.95±0.77a
	B	36.14±0.21b	12.29±0.22b	52.35±2.48a	78.76±1.01a
7	A	42.35±2.28a	14.09±0.26a	52.78±1.52a	74.22±2.30a
	B	46.33±1.55b	16.23±0.24b	54.33±0.97a	71.71±1.50a
15	A	48.35±2.37a	16.73±1.02a	54.81±0.06a	70.25±1.98a
	B	46.42±1.19a	15.82±0.52a	55.30±0.49a	72.08±1.09a
30	A	48.55±2.30a	16.65±0.52a	53.61±1.72a	69.52±2.71a
	B	46.43±0.71a	15.69±0.55a	55.79±2.85a	72.32±0.83a

Results are given as mean ± standard deviation (n = 3); Means in each column with the same letter did not differ significantly (P > 0.05);

The most important biochemical change during ripening of the brined cheeses was the extent of proteolysis which the most influence on the sensory properties of products. The proteolysis parameters of brined cheeses made with commercial and autochthonous starter bacteria were shown in Table 2.

Table 2. Proteolysis paramters of white brined cheeses made with commercial and autochthonous starter cultures during ripening

Day of ripening	Cheese	WSN/TN (%)	PTAN/WSN (%)	PTAN/TN (%)
1	A	3.53±0.22a	8.03±0.32a	0.28±0.01a
	B	4.08±0.09b	11.05±0.46b	0.45±0.01b
7	A	5.39±0.23a	10.92±0.40a	0.70±0.01a
	B	6.14±0.06b	12.76±0.53b	0.79±0.03b
15	A	5.93±0.41a	20.91±0.72a	1.24±0.13a
	B	6.62±0.48b	21.75±0.16b	1.32±0.09b
30	A	6.26±0.21a	20.88±1.05a	1.31±0.09a
	B	6.92±0.30b	22.13±0.66b	1.42±0.06b

WSN/TN – water soluble nitrogen/total nitrogen, PTAN/WSN – nitrogen soluble in phosphotungstic acid/ total nitrogen, PTAN/TN – nitrogen soluble in phosphotungstic acid/total nitrogen, Results are given as mean ± standard deviation (n = 3); Means in each column with the same letter did not differ significantly (P > 0.05);

The content of WSN/TN marked a constant and significantly increases (P<0.05) during all cheese ripening period (Table 2.), but it was the most intense during the first 7 days. The content of WSN/TN was about 7.0% at the end of ripening, which is significantly lower compared to WSN/TN of traditionally made white brined cheese. Abd El Salam (2008) showed that the index of ripening of the most white brined cheeses were within 12–20%. The lower ripening index of experimental cheeses was consequence of short period of ripening. Generally, both cheeses were characterized by a weak proteolysis.

The content of PTAN/TN increased constantly during the ripening period. At the end of the examined period, the content of PTAN/TN was about 1.30% and 1.40% for cheese A and B, respectively. It was significantly lower compared with PTAN/TN of traditionally made cheeses, which is often within 3–5% (Abd El–Salam and Alichanidis, 2008). These results confirm the facts about slower and less distinct proteolysis changes during the ripening of UF cheeses.

Different starter cultures showed significant influence (P>0.05) on the rate of proteolysis determined by WSN/TN and PTAN/RN contents. Autochthonous lactic acid bacteria showed a more intense proteolysis activity probably as result of different proteolytic activity of bacteria used.

Protein profiles of all cheese samples during 30 days are shown in Fig. 1, which indicates no major proteolysis during ripening. As was reported in previous studies (Ong et al., 2006), the β-casein fraction showed only a minor decrease. Intensity of β-casein bands was about the same after storage, showing that neither probiotics nor commercial starters had strong proteolytic activity.

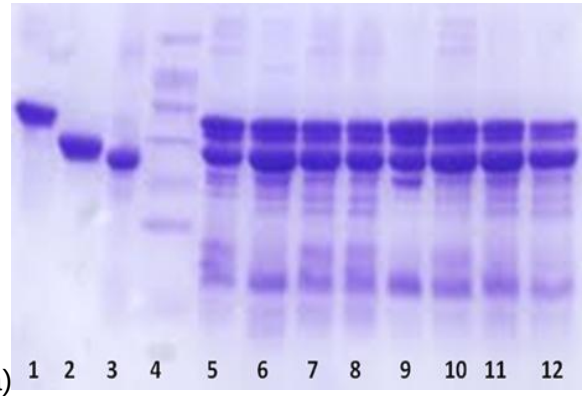
a) 

Figure 1. SDS electrophoretogram of white brined cheeses during ripening
1 - α casein standard, 2 - β casein standard, 3- k casein standard, 4 - standard of molecular weight 14,2-66 kDa, 5, 6, 7, 8 - cheese A and 9,10,11,12 – cheese B after 1, 7, 15 i 30 days of ripening

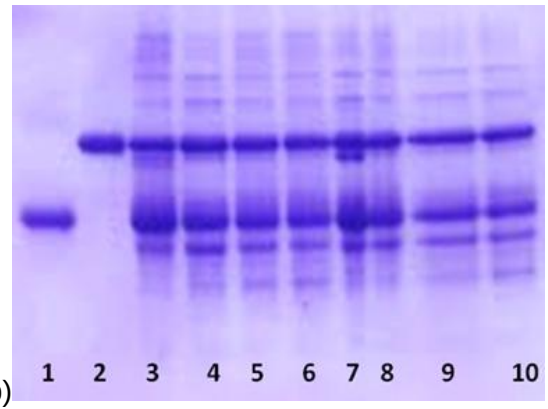
b) 

Figure 1. UREA electrophoretogram of white brined cheeses during ripening
1 – α casein standard, 2 –β casein standard, 3, 4, 5, 6 - cheese A and 7, 8, 9, 10 – cheese B after 1, 7, 15 and 30 days of ripening

CONCLUSIONS

The both white brined cheeses made with commercial and autochthonous starter and adjunct probiotics cultures characterized with very acceptable sensory properties. It can be concluded that autochthonous lactic acid bacteria can use as starter cultures in production of white brined cheese. The application of these bacteria can enable the standardization of traditional made cheeses as well as create a new product with functional properties.

The results also indicate that *Lb. plantarum* 564 is suitable for development of an acceptable probiotic brined cheese. Incorporation of probiotic cultures in cheeses provides potential not only to improve health status and quality of products but also to increase the range of probiotic products as well as improvement of their sensory properties.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III 46009 and 460010 financed by the Ministry of Science and Technological Development, Republic of Serbia.

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PROTECTED GEOGRAPHICAL INDICATION AS A TOOL FOR VALORISATION OF AGRO-FOOD POTENTIALS AND IMPROVING MARKETING: CASE OF "SJENICA CHEESE" IN THE REPUBLIC OF SERBIA

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ABSTRACT: Traditional and typical agri-food products in the Republic Serbia have an important place in consumption. These products, especially from the Pester region in the South-Western of the Republic of Serbia where the Sjenica cheese is produced, have a good image in the Serbian market.
From the market side there are no difficulties for commercialisation of traditional and typical agri-food products. However, only some of them are protected properly, while for the majority there is no protection of any kind and many of them are at risk to be lost from the market and therefore the agri-food potential would be not valorised.
Evidence from other countries, especially from the EU, showed that Protected Geographical Indications (PGI) could ensure proper valorisation of agri-food potential and improve marketing. The objective of this paper was to analyse how PGI of Sjenica Cheese could allow preserving and valorising of resources of Pester region.
Key words: *geographical indications, Sjenica Cheese, agro-food potential, marketing*

INTRODUCTION

Competition in the market of agricultural and food products is permanently increasing. Manufacturers of Serbia still can sell their products due to special taste and quality that are the result of favourable climatic conditions and the specifics of certain localities.
However, most of the agri-food products to both the domestic and foreign market are sold as "no name" and therefore achieve lower prices than products that are trademarks. For producers of Serbia is important to protected designation of origin so to have added value for their products. Otherwise there is a risk that many authentic Serbian products disappear from the market.
The protection of geographical origin is of high importance for small farmers in particular for small family farms in Serbia. In Serbian agricultural production family farms are the most important entities in the aspect of available capacity and in terms of total production. Private family farms use one part of the production for its own consumption, while the remaining part is sold at the market.
Increasing the competitiveness of commercial family farms is one of the strategic goals of Serbia after changes in 2000. In addition, the Ministry of Agriculture holds the position that agricultural and food products are becoming Serbian brand, and that our country has a large, under-exploited production potential. Supporting small family business is in accordance with the EU CAP.
Designation of origin is viewed as a part of marketing instrument and contributes to increasing of the commercial value of products or services. In addition, it is considered that the products with protected origin achieve higher prices (Babcock and Clemens, 2004). Number of products having geographic indication in the EU increases and there is market growth (EU 2007, Mesić et al, 2010).
Numerous case studies show that food quality insurance and certification schemes, including GI, can contribute significantly to the product value. The benefit is limited not only to the farmers and producers, but the other participants in the value chain like wholesalers and

retailers benefit too. Finally, rural areas and society as a whole would enjoy benefits. It is important to point out that benefits should be distributed equally among the participants in the value chain (Gay, 2007).
Aims of the protection of geographic indications of products are to protect product names from misuse, providing information to consumers about the specific features and origin of products, to encourage diversification of agricultural production and rural stability (Soeiro, 2005.).
Protection of geographic indications is in Republic of Serbia regulated by law. Namely Law on Indications of Geographical Origin (Official Gazette of RS No. 18/2010) and Rule book on the content of the request for registration of geographic origin and content requirements for the recognition of the status of authorized user of designation of origin (Official Gazette of RS, No. 93/2010).
Although, almost all Serbian regions are known for some of the products that have a unique recipe, possibilities of protection of agricultural and food products with geographic designations are in Serbia not sufficiently utilized. In Serbia there are 43 designations of origin and each year on average there are two new applications for protection (The Intellectual Property Office of the Republic of Serbia)
This is due to the fact that there is lack of knowledge, information, organizational and marketing skills, financial resources, institutional support and an effective control system. Moreover, studies of social and economic benefits do not exist.
Therefore, the aim of this research is to examine the possibility to valorise the agri-food potential and improve marketing by protection of the geographic indication in the case of “Sjenica Cheese”.

MATERIAL AND METHODS

For this paper the desk research and field visits and interviews with producers using semi-structured questionnaire were applied. Desk research has been focused on collection and analysis of available data on the area and products, while the interviews with the producers aimed to find their perception of protection of geographical indications. The sample size was 49 systematically most advanced family farms from Pester. The data were processed using descriptive statistics and SWOT analysis.

RESULTS AND DISCUSSION

Pester plateau area has favourable conditions for raising cattle, rather than for agriculture and horticulture. Pester plateau abounds in pastures that are distant from the village and are at a considerable altitude. A clean natural environment, rich meadows and pastures, combined with the traditional mode of production have resulted in superior food products characteristic for this area. Farmers from the area have always been known for producing high quality dairy and meat products.
The sheep grown in Sjenica was selected by modification of domestic sheep breeds most common in Serbia. This breed was adapted to conditions of poor nutrition and care, as well as adapting to the frequent rain and snow. It weights on average 45 kg, making it one of the largest Pramenka breed.
Characteristic grasses and herbs to feed sheeps, along with air and water give a special taste and aroma to food from Pester. Authentic conditions of the area such as geographic features and altitude, climate and microclimate factors in the way of cultivation and animal nutrition could not be found in other regions in Serbia.
Sjenica cheese is a dairy product and belongs to a group of white cheese in brine. This cheese is made from sheep, cow or mixed sheep and cow milk, which is characterized by a specific taste, aroma, flavour, texture, and fullness of flavour. The cheese is kept in wooden barrels in which it never deteriorates, as was the case centuries ago.

One of the basic assumptions of the protection of the geographical origin is the use of bottom-up approach where producers, define procedures and practices and provide the majority consent of all interested parties. To undertake activities producers expect to have benefit of activities of geographic indication protection. The main survey findings are presented in the following table. The possible answer were in the range from 1 - do not agree at all to 5 – agree.

Table 1: Producer opinion regarding geographic indications

		MEAN	SD
A	Increase selling price	2,50	0,50
B	Increase volume of selling	3,45	0,82
C	Reduce black market	3,83	0,75
D	Protect misuse of the name	2,00	0,25
E	Increase the confidence of consumers	4,00	0,63

Source: Survey

The result shows that the producers on average do not expect that protection of geographic indications would cause high valorisation of resources. Typical answer is that they do not know if the protection would bring higher product prices. The standard deviation shows that there are no big differences among producers. As the process needs time and money it is to be expected that producers would not protect their product without external support.
It is interesting that there is no expectation that there would be protection of misuse of the name (average answer 2.00). This finding shows that main issues of protection of geographic indications are related to enforcing state regulations regarding property rights. Interesting point is that producers expect that consumer confidence would increase as well as volume of selling. That means that producers are aware of the potential benefit of geographic indications.
In the following table the results of SWOT analysis are summarised providing the most important element for potential valorisation and marketing improvement.

Table 2: SWOT analysis of the “Sjenica cheese”

Strengths	Weaknesses
- Favourable climatic and environmental conditions - biodiversity, climate, altitude - Long production tradition and unique production methods - Positive consumer perception	- Small scale producers - Product packaging not adjusted for small households - Inappropriate marketing skills - Not standardised production process - No appropriate product labelling - Absence of producers’ associations
Opportunities	**Treats**
- Positive trends regarding demand for regional specific product - Institutions recognise the importance of support for the support of the geographic indications - Funds for supporting the process of protection of geographic indications are available	- Opening the Serbian market for supplies from other countries - Serbian consumers do not necessary prefer domestic product more than imported - Government policy is not predictable and therefore for producers is difficult to create strategic plans

Source: Survey

CONCLUSIONS

Republic of Serbia is known by the wide range of the regional products. However, only some of them are protected. Without protection products are marketed as “no name” products. As a consequence selling prices are lower than for products that already have a specific name.

One possibility to increase valorisation of agri-food products is protection of geographic indication.
This was shown on the case of Sjenica cheese. Results of analysis show that producers understand the concept and advantages. However, it could be concluded from the survey that probably this process is not possible without support from the institutions. Regarding marketing, producers expect that with protection consumer confidence in product would increase. In short, solution for valorisation of potential and improving marketing of geographic indicationss products lies not only by producers, but also on the institutions and other members in the value chain

ACKNOWLEDGEMENTS

This paper is the result of the research financed by the Ministry Education and Science of the Republic of Serbia.
Project number: III46001: "Development and implementation of new and traditional technologies in the production of competitive food products with added value for European and world markets - create wealth with Serbian wealth. The research period of this project is from 2011th to 2014th year.

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SPECIFICITY OF GEOGRAPHIC AREA AS ONE OF THE PREREQUISITES FOR THE DENOMINATION OF THE GEOGRAPHIC ORIGIN

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ABSTRACT: Defined geographic area with their specifity regarding unique plant species and autochthonous breeds – Sjenica and Pirot Pramenka, among traditional cheese-making practice of local community, are prerequisite for protection of denomination of geographic origin of Sjenica cheese and Pirot kachkaval.
Sjenica cheese belongs to the group of soft, white cheeses in brine and it is produced as autochthonous product made of sheep milk, on the territory of Sjenica-Pester Plateau.
Pirot kachkaval is firm cheese of steamed curdle produced from mix of sheep and cow milk (1 : 2 ratio) on the territory of Stara Planina Mountain.
These are geographical areas where production takes place in village households/farms and on mountains in summer cottages (katun, bacija), which is characteristic of craft dairy processing. Specific traits of traditional manufacturing of Sjenica cheese and Pirot kachkaval derive from climatic conditions, characteristic grass associations of the specific mountain region and milk from autochthonous populations (Sjenica and Pirot sheep). In order to maintain the traditional manufacturing technology of these native products and to have organized production, it is necessary to protect the geographical origin of these products.
***Key words**: specificity of geographic area, Sjenica cheese, Pirot kachkaval*

INTRODUCTION

European Community, in its Regulation (EEC) No. 2081/92, has defined the concept of »Protected Denomination of Origins« (PDOs) – denomination of origin, and »Protected geographical Indications« (PGIs) – geographic indication for products whose quality derives from the uniqueness of the geographic area of their origin, including human and natural factors, and which are produced and processed in defined, unique geographic areas (Mijačević et al., 2005).
From this aspect we analyzed the specificity of thge geographic area, its pedological, climatic, floristic characteristics and breed structure in sheep breeding, for the purpose of obtaining of the denomination of the geographic origin for Sjenica cheese and Pirot kachkaval.

MATERIAL AND METHOD

Investigation included survey of the traditional production of Sjenica cheese and Pirot kachkaval, which comprised survey of 12 households/farms on the territory of Sjenica-Pešter Plateau and Stara Planina Mountain, randomly selected. Survey consisted of questions relating to main elements of traditional production of Sjenica cheese and Pirot kachkaval. On Pešter Plateau the following locations were selected: Štavalj, Karajukića bunari, Krstač, Breza, Leskova, and on the territory of Stara Planina mountain, the study was carried out in following villages: Dojkinci, Brlog, Jelovica, Visočka Ržana and Rsovci. Eventually, the following analyses of samples of Sjenica cheese and Pirot kachkaval were carried out:

- Determination of the water content in fat free matter of cheese by using the following calculation: %WFFMC=%H_2O/(100-%MM)x100
- Determination of the milk fat content by method according to Van-Gulik (Carić et al.,2000)
- Calculation of the milk fat content in dry matter (FinDM)
- Determination of pH value by using the pH-meter with combined electrode.

RESULTS AND DISCUSSION

Characteristics of Sjenica – Pešter Plateau and conditions for autochthonous production of Sjenica cheese

Sjenica – Pešter Plateau is located in the south-western part of Raška region and in the north it occupies smaller part of Old Vlah. In the east it borders with Novi Pazar low land and Donji Pešter, in the south with lower Kolašin, and in the west with central Polimlje. It is surrounded with mountain ranges of Golija, Javor, Jadovnik, Ozren, Žilindar, Ninaja and Pometenik. This area represents a unique mountain range, with numerous valleys, ravines, fields, which are located between 1000 and 1350 m above sea level. Pešter is an area with typical carst field and morphological forms which are characteristic of carst: places where rivers sink into earth, lost rivers, waters and caves – so called “pešters“.

This Plateau has continental climate of typical of mountain Plateaus, characterized by long retention of low temperatures between the surrounding mountains, and winters last very long time, because of poorly developed forest strips, with lot of snow and wind, with strong snow storms and great snow drifts. Summers are fresh, short lasting and windy with distinct difference in temperatures between day and night time. Geological foundation consists of gravel, sand and clay, which from the aspect of vegetation structure indicates that meadows and pastures are predominant.

In the area of Sjenica-Pešter Plateau, main branch of economy is agriculture, i.e. livestock production, dairy farming. Mainly sheep (approx. 18000 heads) and cattle (total of 16437 heads, of which 12000 are cows) are reared (Petrović, M. M, 1993). Dominant sheep breed is Sjenica sheep. This is the largest Pramenka, of triple production abilities, it has white colour fleece all over its body except on the face and lower legs. The body is medium-developed, deep with insufficiently expressed widths. Head is of medium length with typical black circles around eyes. Milk performance ranges from 80-130 l in lactation of 180 days. This sheep breed is suitable for rearing in all areas and systems, extensive and intensive, it can be used in production of milk and meat (Petrović, P. M., 2007).

Rearing of livestock is different in winter and summer. In summer, livestock grazes on meadows and pastures which are either state owned or in private ownership. After the vegetation period, from November to May, nutrition of sheep is based on hay from meadows (grass-leguminous mixtures), where high quality cultures are predominant such as Timothy grass, rye grasses, alfalfa, red clover, etc. In addition to hay, during winter, also concentrated feeds are used which livestock farmers prepare them selves by grinding the cereals/grain (barley, oats, wheat) produced on their own field crop surfaces. Structure of plant associations in this area has significant impact on the specific quality of Sjenica cheese. Table 1 presents floristic composition of pastures on location Vrujci, on Sjenica – Pešter Plateau, 1158 m above sea level.

Share of plants belonging to plant family Poaceae – grasses on this location was 48,40%, and shares of leguminous plants and other species were 9,60% and 42,00%, respectively. In case of grasses, the highest share was determined for plants of good and medium quality, from the aspect of livestock nutrition.

Table 1. Floristic composition of pastures on location Vrujci, on Sjenica – Pešter Plateau (Vučković et al., 2004)

Fabaceae,%	Poaceae,%	Other species,%
Genista sagittalis *Lathyrus latifolius* *Lotus corniculatus* *Trifolium pratense* *Vicia cracca* *Trifolium alpestre* *Trifolium panonicum* *Trifolium montanum*	*Cynosurus cristatus* *Anthoxantum odoratum* *Arrhenatherum elatius* *Briza media* *Danthonia calycina* *Bromus raceomosus* *Agrostis vulgaris* *Dactylis glomerata* *Festuca rubra* *Festuca ovina* *Phleum pratense*	*Achilea milefolium* *Alectrolophus minor* *Cirsium acaule* *Dianthus deltoides* *Filipendula hexapetala* *Galium verum* *Leucanthemum vulgare* *Moencia mantica* *Poentilla recta* *Ranunculus sp. Rumex acetosa* *Stellaria graminea* *Thymus serpyllum* *Leontodon autumnalis* *Silene vulgaris* *Stachus officinalis* *Plantago lanceolata* *Campanula patula*
Total,%	Total,%	Total,%
9.6	**48.4**	**42.0**

On defined/determined geographic area of Sjenica – Pešter Plateau, which is specific in regard to climate, phytocenological composition of pastures, sheep population and tradition, the Sjenica cheese is produced. It belongs to the group of soft, white cheeses in brine, and it is autochthonous cheese produced from raw sheep milk. Production takes place in households/on farms and also in the mountains in so called summer cottages (katuns, bačije), which is typical of home production and processing of milk. Since sheep are kept around these summer cottages from May to November, ripening process of Sjenica cheese lasts from 5 to 7 months. Low pH value and long ripening process prevent development of pathogen microorganisms and contribute to safety of this cheese. In Table 2, the chemical composition of Sjenica cheese is presented.

Table 2. Physical-chemical composition of Sjenica cheese

Parameters	**MFFB,%**	**FDM,%**	**pH**
Number of samples	12	12	12
X	73.32	58.42	4.47
SD	2.9	1.74	0.07
CV	4.30	2.30	1.5

Legend: **MFFB** *– water in fat free matter; FDM – fat in dry matter*

Data presented in Table 2 show the average water content in fat free dry matter of cheese (MFFB) of 73,32% %, hence it belongs to the group of soft cheeses, and in regard to content of fat in dry matter (58,42%), it belongs to the group of full fat cheeses (Codex Alimentarius, 2000). Average pH value was 4,47 indicating acid environment which is not suitable for development of pathogen microorganisms. Analyzed cheese samples had adequate sensory characteristics: cheese slices of adequate form, no deformities or damages, and typical white colour. Cheese slice was typical, medium firm, and cross section had moderate number of holes. Odour/smell was also typical, clearly indicating sheep milk, and taste was pleasant and dairy – sour. Since Sjenica cheese is characterized by a distinctive and recognizable

richness of the taste, aroma, odour, this product is in demand and appreciated outside the territory where it is produced.

Characteristics of Stara Planina Mountain and conditions of autochthonous production of Pirot kachkaval

Stara Planina Mountain constitutes far western branches of the Balkan mountain system, stretching from the Black Sea to Vrška čuka, the total length of 530 km. In Serbia, it extends with its slopes from Zaječar to Dimitrovgrad, covering the far eastern part of Serbia. In geological aspect, all formations are represented on this mountain, from Palaeozoic to the most recent formations. In completely defined geographic area/territory of Stara Planina Mountain (Pirot, Dimitrovgrad), in conditions of specific climate, phytocenological composition of pastures, Pirot kachkaval is produced using the milk from Pirot sheep.

Climatological picture of Stara Planina Mountain is very complex and, depending on the altitude, is represents transition between moderate continental and mountain climate. Diverse geological-petrographic, morphological, climatic, hydrographical and other factors have influenced forming of different pedological types of productive soil, with various physical, chemical and biological properties.

The diversity of geographical and ecological/environmental factors of Stara Planina Mountain has caused the specificity of its plant and animal life (Petrović, 1997).

Table 3 shows the presence/share of plant species in pastures of certain localities of Stara Planina Mountain.

Table 3. Presence/share of plant species in pastures of Stara Planina Mountain (Tomić et al., 2003)

Location (a.s.l.)	Fabaceae,% Leguminous plants	Poaceae,% Grasses	Other species,%
Rsovci, 700m a.s.l.	*Trifolium pratense* *Trifolium repens* *Lotus corniculatus* *Lathurus sativus* *Vicia sp.* **39,36**	*Festuca rubra* *Festuca ovina* *Lolium italicum* *Poa violacea* **45,81**	**14,83**
Vrelo, 750m a.s.l.	In traces	*Anthoxantum odoratum* *Festuca arundinacea* *Festuca ovina* *Lolium italicum* *Agrostis capillaris* **78,80**	**21,20**
Brlog ,800m a.s.l.	*Trifolium pratense* *Trifolium repens* *Lathurus sp.* *Vicia sp.* **42,40**	*Nardus strictae* *Festuca arundinacea* **44,00**	**13,60**
Dojkinci,900m a.s.l.	*Trifolium repens* *Trifolium pratense* *Trifolium campestre* **15,60**	*Festuca ovina* *Nardus strictae* *Agrostis capillaris* **47,80**	**36,66**

In the floristic composition of the pasture, leguminous plants represent the group of plants very important for animal nutrition and increase of production of milk and meat. The following useful leguminous plants determined in studied areas were most represented: Trifolium pratense, Trifolium repens, Trifolium campestre, Trifolium montanum, Lotus corniculatus, Vicia craca, etc., and their share ranged from 0 to 42,40%. Share of grasses ranged from 44,00-82,31%, whereas other plant species where presented in the range from 13,60-36,66%.

As the result of exceptional natural conditions, livestock production has centuries old tradition. In the structure of animal population, due to specific natural and ecological/environmental factors, sheep breeding has the leading position. The most famous products from this unpolluted area are: Pirot kachkaval, Pirot lamb and Pilot kilim (Petrović, P. M, 1993). First »wheels« of Pirot kachkaval set out into World were manufactured on Stara Planina Mountain (Petrović, 2007). Mastery of making kachkaval in this area dates from the time of nomad livestock keepers, so called «Crnovunci« who lived on the pastures of Stara

Planina Mountain from the end of the 19th century until the third decade of the twentieth century. Indigenous production of Pirot kachkaval is the result of many years of developing of the production technology through generations.
The specificity of this autochthonous product (Pirot kachkaval), in addition to climate, soil, botanical composition of pastures and meadows, is under the considerable influence of sheep population, whose milk is used as raw material for manufacturing of said products, as well as rearing conditions (Štimac et al., 2003). In regard to sheep breed structure, predominant breed is Pirot Pramenka. This is an old sheep breed adapted to conditions of Stara planina Mountain and Pirot area, which, in time, and due to improved nutrition and rearing, has realized significant genetic progress. Pirot Pramenka is breed of triple production ability, it has fleece of white colour all over the body, except its face and lower parts of legs. The body is medium-developed, with insufficiently expressed widths and depths. The head is of medium width and length. Hair covering the face is white or grey-spotted. Milk performance is good and it ranges from 70-100 l in lactation of 180 days. (Petrović, P. M., 2007). Quality of sheep milk among other things depends on the climatic conditions, grazing and variations caused by metabolic status of the sheep (Sevi et al., 2004). Also, yield and chemical composition of milk depend on the genotype, lactation stage and health condition of animals (Mioč et al., 2009). Pirot kachkaval is produced from mixture of raw sheep and cow milk (ratio 1: 2). Production takes place in settlements high in the mountains, so called »bačije« according to traditional technology. It is cheese from steamed curdle and technological procedure, in addition to standard stages (curdling, curdle treatment), there are also following stages: ripening and steaming of ripe curdle which takes place at t^0 85-90^{0}C, in duration of several minutes. This heat treatment of curdle prevents the development of undesirable microorganisms. The quality of Pirot kachkaval obtained in this way, expressed in physical-chemical parameters, is presented in Table 4.

Table 4. Physical-chemical characteristics of Pirot kachkaval

Parameters	**MFFB,%**	**FDM,%**	**pH**
Number of samples	12	12	12
X	47.07	48.43	5.46
SD	1.30	1.82	0.03
CV	2.70	3.20	0.50

Recently, number of cheese consumers who demand high quality exclusive products produced in small quantities and by implementation of „traditional“ technology has been increasing. Cheeses produced in this way have richer aroma compared to industrial cheeses produced according to strictly defined technology and in controlled production conditions, therefore the influence of raw material used, micro flora and creativity of the producer are reduced to minimum (Štimac et al., 2003). Therefore, indigenous production of Pirot kachkaval is of great importance for domestic and foreign market. In order to maintain and preserve the traditional technology and have organized production, it is necessary to protect the geographic indication of these products (Jovanović et al., 2004).

CONCLUSION

Determination of the geographic area of the Sjenica-Pešter Plateau and Stara planina Mountain, as well as specificity of these areas due to micro-climatic factors, terrain and geological characteristics, structure of the vegetation and phytocenological composition of the pastures, as well as breed structure and existing tradition in cheese making, are arguments for obtaining of the geographic indication of origin for Sjenica cheese and Pirot kachkaval.

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ANALYSIS OF MASS AND ENERGY BALANCE IN THE INITIAL STAGE OF KAJMAK PRODUCTION

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ABSTRACT: Kajmak is a traditional Serbian dairy product which can be classified between cheeses and butter. Initial stage of kajmak production (first 60 min of kajmak formation process) results in surface coagulation of top layer of milk, due to mutual influences of: (1) surface tension driven by protein concentration increase; and (2) water evaporation driven by high milk temperature.
In traditional production, milk temperature is decreased for 30-40°C during the initial phase of kajmak formation. Skin layer, initially formed, incorporates only about 10-15% and 2-3% of total milk fat and proteins, respectively. Numerical simulation of the kajmak formation process indicated the existence of the energy loss, which is mainly caused by the complex processes of simultaneous convective and evaporative cooling of milk.
Creation of the modern procedure for kajmak production faced the request for changes regarding both, mass and energy balance. Analysis of the mass balance of the kajmak production indicates that it is possible to complete the initial phase of kajmak production with significantly reduced amount of milk. Adversely, energy balance analysis indicates that the reduction in the amount of milk at the same time creates a serious energy deficit. Therefore, one of the central issues of modernization of kajmak production is addressed to the adequate balance of mass and energy.
Key words: *kajmak, surface activity, mass and energy balance, convective and evaporative cooling*

INTRODUCTION

Kajmak is traditional Serbian dairy product usually produced in households and small dairy plants. Kajmak, due to its specific properties, can be (clasified) between cheeses and butter. Characteristics of kajmak show great variation *(Dozet et al., 1996; Pudja et al., 2005b)*. This is mostly caused by variation of milk composition during lactation, type of dairy cows diet, as well as by changeable environmental conditions during kajmak production.
Process of kajmak formation can be divided into two phases: (i) hot and (ii) cold phase. During the hot phase the initial kajmak skin is formed, and it represents kajmak's upper layer. This layer is then, in the cold phase, being enriched with milk fat, and thus the lower layer of kajmak is formed *(Pudja et al., 2004)*.
Parameters influencing formation of upper and lower layer are at phase distance and the natures of their driving forces are completely different. In that respect, their characterization and optimization may be carried out separately. Traditional method of manufacture occupies very long time, and contains numerous steps which make up potential safety and quality risks (*Djerovski et al., 2006).* In order to carry out the industrialization of kajmak production it is necessary to standardize all steps of manufacturing process. This ultimately leads to risk-free product with standard high quality.
Fact that initial skin incorporates less than one-third of milk fat of whole kajmak suggests that its formation can be performed with relatively small amounts of milk (*Puđa et al., 2005a*). As a result of high surface tension, surface-active milk components concentrate at milk-air interface *(Kristensen et al., 1997)*. Formation of initial skin is a result of protein coagulation at milk surface on account of enormous rise of their concentration, whilst the greatest extent of dry matter of initial skin is milk fat.

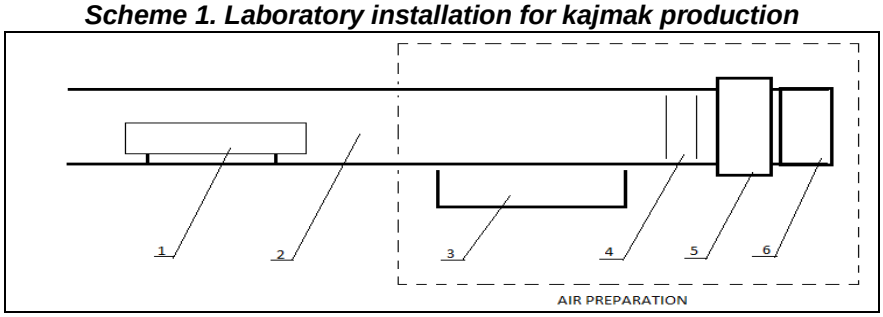
Troughout the formation of kajmak surrounding air remains in contact with milk, and afterward with the initial skin. Temperature and relative humidity are the key factors determining process course and therefore initial skin and kajmak properties (*Pudja et al., 2004*). Function of air is conveying water and energy away from the system. Hence, better defining of mechanism and dynamics of mass and energy conveying during the procces of kajmak formation is one of the central issues of industrialization of kajmak production.
Our objective was to examine mass and energy balance during the formation of initial kajmak skin, by applying different conditions regarding air parameters and amount of used milk.

MATERIAL AND METHODS

Production of initial skin

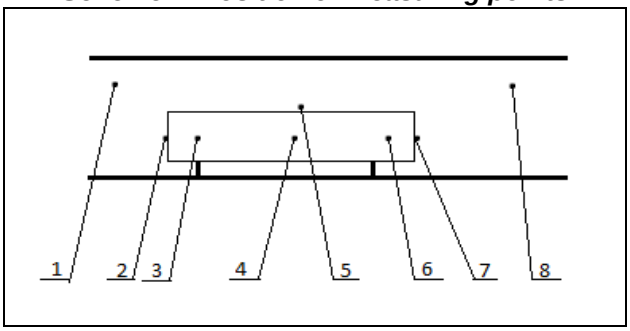
This paper investigates first 60 min of kajmak formation from milk of average composition (4% fat, 3.4% proteins). Raw milk was obtained from Dairy plant „PIK Zemun“, Belgrade. Heat treatment of milk included heating to 95^0C, with detaining holding for 10min at 85^0C and 10min at 95^0C. Following heat treatment, milk was placed under specific conditions and initial skin was being formed. In that purpose, vessels P1 and P2 were used, with indentical base dimensions 0.2x0.43m. Vessel P1(wall height 0.10 m), with milk level of 0.08 m is suitable for simulation of traditional method of kajmak production, while vessel P2 (wall height 0.05 m) with milk level of 0.03 m used suits recommended industrial procedure.
Initial skin formation was examined through experiments A, B and C. Experiment A was conducted using P1, under conditions reflecting those in traditional method of manufacture where milk was subjected to enviromental air. Experiments B and C were conducted in test instalation for kajmak production, with invariable air parameters (30ºC; 70% RH, 0,7 m/s). This instalation consists of 0.2x0.3m tunnel and elements for air preparation (Scheme 1). Experiments B and C were performed in P1 and P2 vessels, respectively. Parameters of inlet and outlet air and milk used were measured at points indicated (Scheme 2). Digital thermometers Peack Tech 5110/5115, Germany, was used to measure temperature of milk and temperature of vessel wall, while air parameters were measured with Kestrel 4000 NK instruments, USA.

Scheme 1. Laboratory installation for kajmak production



1. Vessel; 2. Tunnel; 3. Humidifier; 4. Heater; 5. Cooler; 6. Fan or ventilator

Scheme 2. Position of measuring points



1. T_1, RH_1 – inlet air parameters; 2.,7. T_2, T_7 – vessel wall temperature; 3.,4.,6. T_3, T_4, T_6 – milk temperature; 5. T_5 – temperature of air streaming over milk; 8. T_8, RH_8, V_8 – outlet air parameters.

Milk heat loss, i.e. heat flux caused by evaporation and heat transfer trough vessel walls, was calculated using measured air parameters (*McAdams., 1954; Mills., 1995; Burmeister., 1993; Voronjec and Kozić., 2002.).*

RESULTS AND DISCUSSION

Analysis of mass and energy transfer

During formation of initial skin air flows over the milk surface establishing heat and mass transfer. At interface, milk and air particles have equal temperature, regardless temperatures of steady airflow away from milk and milk at certain depth.

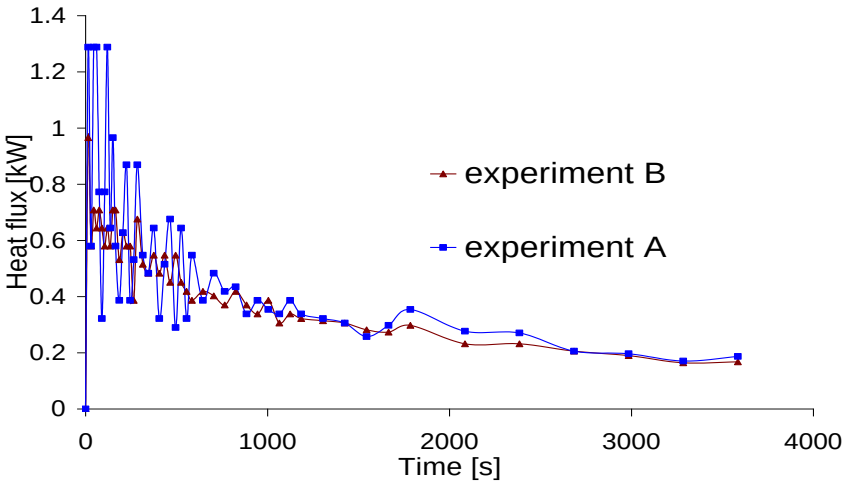
Dalton law states that amount of evaporated water from milk at milk-air interface is equivalent to difference in absolute humidity of interface air and steady flow air. Convective cooling of milk is conditioned by temperature difference of steady flow air and interface air.

At the beginning of process, part of heat flux is transferred to airflow by evaporative milk cooling. Consequently, airflow receives certain amount of vapor and meanwhile airflow temperature is increased owing to convective cooling at interface. Considering the fact that milk vessel is positioned in tunnel where all its sides are in contact with airflow, milk is additionally cooled by heat transfer trough vessel walls. This process of coupled evaporative and convective cooling causes faster milk cooling at interface and in case of small amount of milk resulting in process time decrease.

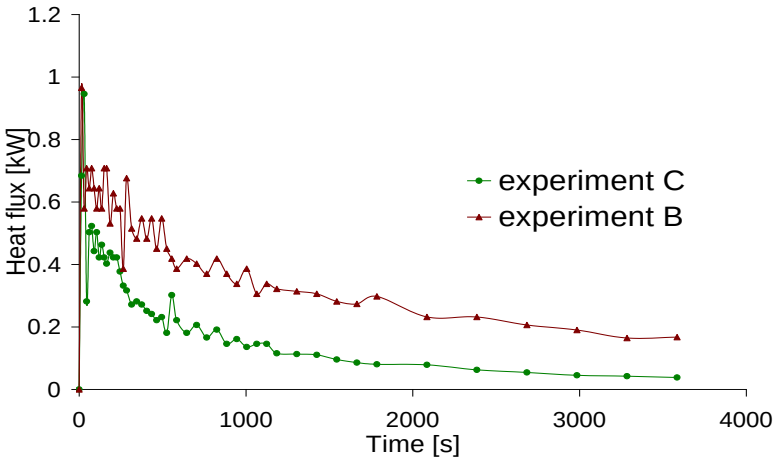
After formation of initial skin, heat flux from milk to air is established in form of heat transfer trough vessel walls and formed initial skin. Heat transfer trough vessel walls is continuous process, but intensity of exchanged heat flux decreases over time due to gradual cooling of milk and decrease of temperature difference between milk and air. In the case of heat transfer trough the initial kajmak skin layer at milk/air interface it can be assumed that convection, both from air and milk side is more dominant than conduction trough the kajmak skin layer. Since the thickness of initial skin in the initial phase is low, this part is negligible, but in later phases, due to accumulation of material it needs to be taken into account when heat balance is analyzed. Water evaporation from milk is present throughout the milk cooling process due to kajmak skin layer porosity and is taking place parallel with heat transfer trough the plate walls and skin layer. Water evaporation into free airflow is continuously present, but amount of vapor decreases over the time as milk temperature and skin porosity reduce.

Mass and energy balance

Data of energy consumption during the first 60 minutes of kajmak production, which was acquired from experiments of traditional kajmak production process with uncontrolled conditions and simulation of innovative procedure of kajmak production in controlled conditions are shown in Figure 1.



a)



b)

Figure 1a. Energy consumption during the initial kajmak skin formation with same amount of milk with uncontrolled (A) and controlled (B) air parameters

Figure 1b. Energy consumption during the initial kajmak skin formation with different amount of milk and constant air parameters

Examining traditional kajmak production gives us that air temperature and relative humidity are dependent of environmental air parameters and room natural ventilation. Calculation of evaporative heat flux in initial stage of traditional kajmak production is impossible due to the fact that vapor at milk/air interface is intercepted and absorbed by large amount of environmental air. This amount of vapor is negligible comparing to the amount of air in the room, thus relative humidity of air in room would not be changed.

Also, the presence of very unstable heat transfer from milk during initial phase of kajmak formation was found. This can be explained with changeable air parameters and local air speed around the vessel in this stage. After the skin is formed, when heat transfer from milk gets generally convective character, energy consumption becomes more stable. Comparing traditional production with simulation of modern kajmak production under the constant environmental conditions (Figure 1a), it can be concluded that energy consumptions in both cases have similar character. Simulation of modern kajmak production is carried out with higher inlet air temperature and with less air compared to amount of air in traditional production. Energy consumption during the kajmak formation in controlled air conditions but with different amount of milk was very similar which can be seen in Figure 3b.

Total heat flux observed in 60 min of milk cooling is very dependent of milk level, i.e. unt of milk used (Table 1). Reduction of milk level from 8 cm to 3 cm resulted in total heat flux decrease of 42%. However, when energy consumption is expressed per kg of milk it can be found that there is a notably higher consumption (5.11 kW/kg) for vessel with lower level of milk (experiment C), comparing to 3.67 kW/kg for vessel with higher level of milk (experiment B). Comparison of experiments A and B, that were performed with identical level of milk, but with different air parameters, demonstrates insignificant difference in energy consumption. This points out that choice of appropriate air parameters can move modern industrial production towards traditional method.

Table 1. Overall heat consumption during the skin layer formation (60 min.)

Experiment	Inlet air temperature [°C]	Inlet air φ [%]	Milk, height of column[m]	Q [kW]
A	15 - 17	43 – 45	0.08	25.15
B	30	70	0.08	22.01
C	30	70	0.03	12.78

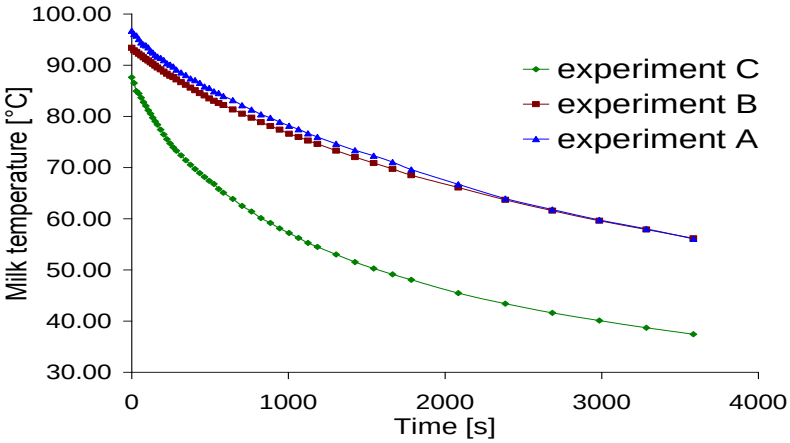


Figure 2.Change of milk temperature during the kajmak skin layer formation (60 min.)

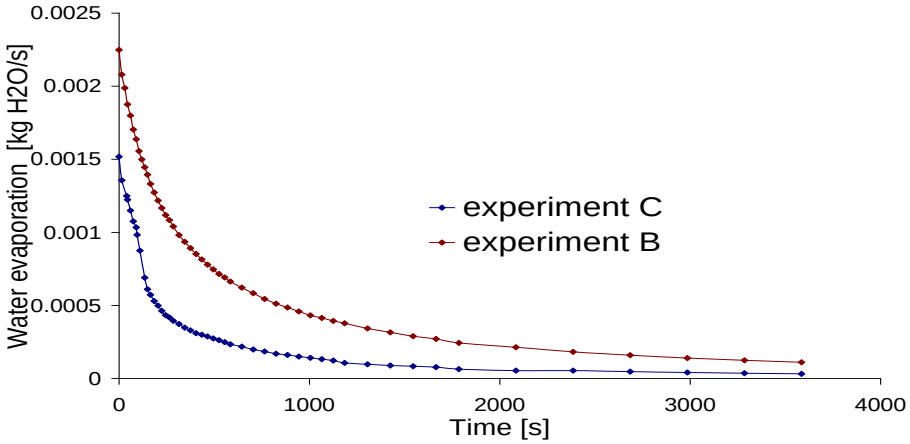


Figure 3. Water loss caused by water evaporation during the skin layer formation (60 min.)

Course of milk temperature change in experiment A is very similar to temperature change in experiment B. Also, milk temperatures in these experiments, are from the moment of establishment of stationary conditions, almost identical.

Formation of the initial kajmak skins in experiments performed with different levels of milk used (B and C) showed that temperature changes were more noticeable in vessel with lower level of milk. This is probably result of faster cooling of lesser amount of milk used (experiment C).

Based on the results showed above, it can be concluded that reducing od milk amount as well as height resulted in energy deficit.

All of this mentioned earlier, leads to conclusion that reducing of milk level results in energy deficit which must be compensated, and thus approach to traditional method of manufacturing.

Comparing traditional production with simulation of modern kajmak production concept under the constant environmental conditions (Figure. 3), it can be concluded that the water evaporation process was very intensive and showed similar character regardless the amount of milk used. In later phase of process, the evaporation was reduced as a result of skin formation and milk cooling process.

Evaporation was stabilized during the final stage of the kajmak skin formation process which was the result of the stable mass transfer through the skin formed.

However, it can be concluded that the amount of water evaporated varied depending on the milk level, i.e. amount of milk used. The loss of water, i.e. amount of 1.51 and 0.57 kg in experiment B and C, respectively, was found despite the same surface and environmental conditions. Difference found in quantity of evaporated water was a consequence of lower heat capacity of milk used.

CONCLUSIONS

Analysis of the mass balance of the kajmak production indicates that it is possible to complete the initial phase of kajmak production with significantly reduced amount of milk.
Similar changes of heat flux during the traditional kajmak production and modern procedure performed with same milk level but different environmental conditions was found.
Lowering the milk level leaded to serioius energy deficit which furthermore contributed to faster cooling of milk and lesser intensity of evaporation. Theese results indicate that it is necessary to compensate mass and energy deficit.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project No. 46009 financed by the Ministry of Education and Science of the Republic of Serbia.

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PROXIMATE COMPOSITION AND SENSORY CHARACTERISTICS OF SREMSKA SAUSAGE PRODUCED IN A TRADITIONAL SMOKING HOUSE

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ABSTRACT: The goal of this paper was to determine the proximate composition and sensory characteristics during storage of Sremska sausage produced in a traditional way in the smoking house, from the meat of commercial 7-month-old Swedish Landrace pigs. Sausages were produced in December; the muscle-fat ratio was 75:25, with the addition of 2.2% nitrite curing salt and spices. After filling in pig small intestine, the sausages were hung to dry with the temperature ranging between 10–15^{0}C and relative humidity between 75–90%. They were then occasionally smoked during the first 14 days. Sampling and chemical analyses were carried out on 0, 3rd, 7th, 14th and 21st day of production. Sensory evaluation was performed at the end of the production process (first day) and during storage, on 60th and 120th day. The sausage was characterized by a long ripening period, which is considered typical for the traditional production method. The minimum pH (4.97) was reached on 14th day, then it increased slightly to 5.18 (21st day). The values of acidity and peroxide number of the fat increased at the end of the production process. During the ripening, water content significantly decreased (from 58.13% at beginning to 30.21% at the end), that resulted in large weight loss of 41%, while the proportion of fat, protein and ash significantly increased. At the end, final product had distinctive spicy flavor, dark red color and firm consistency. During storage, overall sensory quality has slightly reduced, but sensory scores were still high for this type of product.
Key words: *Sremska sausage, traditional smoking house, proximate composition, sensory characteristics*

INTRODUCTION

Majority of fermented sausages are produced in Italy, Germany and Spain (Di Cagno et al., 2008), but almost every European country has at least several country specific products belonging to this group (Casaburi et al., 2007). Sremska sausage is typical representative of fermented sausages of small diameter in Serbia, and it is traditionally produced in households or small manufacturing facilities in the region of Vojvodina (North Serbia). In households, traditionally, during winter time, Sremska sausage is produced from pig meat, with the addition of salt and spices. Stuffing obtained in this way is often filled using manual stuffer into pig intestines which are then formed into pairs 20-25 cm long. Sausages are hung on wooden sticks, drained, and transferred to smoking room where they are smoked using so called cold procedure for 6 to 7 days, often even longer. After smoking process, comes drying which usually lasts about 2 weeks (Tojagić, 1996). In said conditions, maturation is finished by the end of the third week, when Sremska sausage acquired all typical sensory and physical-chemical properties. Said composition and production conditions (animals fed in households with various food, smoking of sausages in conventional smoke rooms, air drying) are some of the factors of the specificity of the sausages produced in this manner, and give them advantage in regard to sensory traits, compared to sausages produced in industrial conditions where climate chambers are used (Karan et al., 2009).
Currently, there is a much higher market share for the sausages produced with starter cultures and in controlled climatic conditions than the naturally fermented sausages. However, there is a strong demand for the naturally fermented sausages by consumers. In

this regard, objective of this paper was to determine changes in chemical composition during production and of sensory properties during storage of Sremska sausage produced in traditional smoking house.

MATERIAL AND METHODS

The sausages were produced during December in small processing plant of the Institute for Animal Husbandry (Belgrade, Serbia). In production of sausage meat originating from pigs of Swedish Landrace of average age of 7 months, was used. Main raw materials used for production of Sremska sausage were meat of leg and shoulder and back fat in ratio 75:25. Meat and fat tissue were previously frozen at the temperature -18^{o}C, and then chopped using the cutter machine (Seydelman K60) until granulation of around 8 mm was achieved. Following additives were used: 2.2% nitrite salt, 0.3% sugar (glucose), 0.8% hot ground peppers, 0.5% sweet ground peppers, 0.15% onion (powder) and 0.15% black pepper (ground). Stuffing was filled into pig small intestine, diameter 32 mm. After filling, sausages were hung in the traditional smoke room, without any possibility of temperature and humidity control, where temperature ranged from 10 to 15^{o}C and humidity from 75 to 90%. Sausages were occasionally smoked during first 14 days. Whole process of production lasted 21 days, after which time sausages were vacuum packaged and stored at 4^{o}C. Samples used in our study were taken on 0 (mix before stuffing), 3rd, 7th, 14th and 21st day of production and on 60th and 120th day of storing.

During the production process the following was monitored: weight loss, by measuring 12 individual sausages on a scale (Chyo MK-2000B), accuracy of 0.1 g; pH value, measured using pH-meter with glass electrode Hanna, HI 83141 (Hanna Instruments, USA); the values of acidity and peroxide of the fat were determined, following the Serbian Official Standards SRPS ISO 660 2000 and SRPS ISO 3960 2001, respectively, after extraction of the fat, following the method of Folch et al. (1957); main chemical composition: quantity of water by drying of samples at temperature of 105^{o}C to a constant mass, quantity of fat by method of extraction according to Soxlet (with petrol-ether as solvent), quantity of proteins using method according to Kjeldahl and quantity of mineral matters (ashes) by burning of samples at temperature of 550-600^{o}C to a constant mass (AOAC, 1990).

Sensory analysis of sausages was done by seven semi-trained evaluators at the end of production process (first day) and after 60 and 120 days of storage. For sensory evaluation numeric-descriptive scales with 5 point system were used (from 1-exceptionally unacceptable to 5-exceptionally acceptable). Following meat attributes were determined/scored: appearance, colour, texture, odor and taste.

For statistical analyses of changes in proximate composition during production process the ANOVA procedure of Statistica 7 software (StatSoft, USA) was performed. If the main effect was found significant, Tukey's test was used to evaluate the significance of difference at $p<0.05$.

RESULTS AND DISCUSSION

The drop of pH value in traditional dry sausages is slight and fermentation is long because of low temperatures (Stajić et al., 2011). pH values are result of the influence of multiple factors, the most important are the amount and type of added sugars, type of muscle tissue and composition of stuffing, as well as present microflora (Hiero et al., 1997). Changes in pH values of Sremska sausages during ripening are given in Figure 1. During the first two weeks of ripening, the pH value decreased from 5.88 to about 4.97, possibly due to production of organic acids by microorganisms, basically production of lactic acid (Lücke, 1994). After that, it increased slightly to about 5.18 in the last 7 days of ripening, probably as a consequence of proteolytic changes. These results were in agreement with literature data for similar fermented products (Salgado et al., 2005; Kozačinski et al., 2008; Stajić et al., 2011).

Processing conditions can influence the rate of acid production and also the change of pH value in products (Salgado et al., 2005). In the research of the changes occurring during ripening of Sremska sausage in climate chamber, Živković et al. (2012) stated that the minimum pH value was reached on day seven of the production process, afterwards a slight increase was registered, to reach the value of 5.17 at the end of production process (14th day).

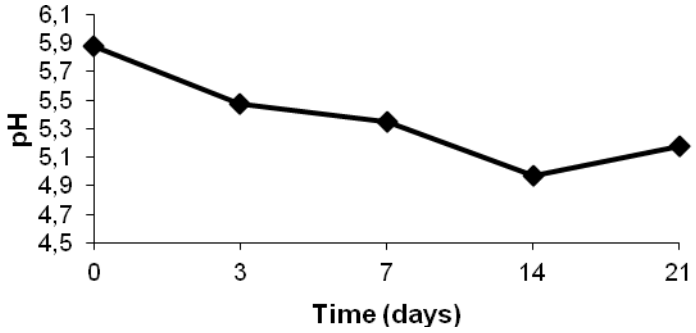

Figure 1. Changes in pH value during production

Dry fermented sausages are characterized by significant water loss during production process, whose intensity depends on the duration of drying, air temperature and humidity (Lücke, 1994). Figure 2. shows the weight loss during production of Sremska sausage. The most intensive loss occurred between day 3 and 7 of production. After one week, the weight loss was 25.25%, and at the end of the production process 44.05% (21st day).

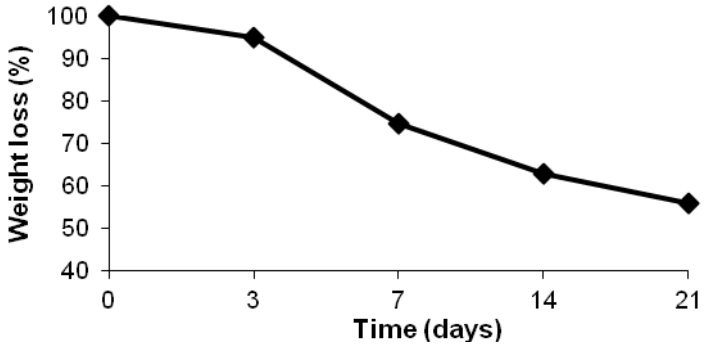

Figure 2. Weight losses of Sremska sausage during production (%)

The acidity of the fat increased during the ripening of Sremska sausage (Fig. 3). The acid number increased slightly in the first seven days of ripening and, afterwards, more rapidly until the end of ripening. Increase of the acid number during ripening of fermented sausages was recorded by other authors, however, our final value at 21st day (8.11 mg KOH/g fat) are much higher than those described by (Roncalés et al., 1991; Salgado et al., 2005).
The intensity with which lipolysis is developed during ripening varies considerably between different varieties of fermented sausages as a function of the different manufacturing procedures and the raw material used (Lois et al., 1987; Domínguez Fernández and Zumalacárregui Rodríguez, 1991). The peroxide number increase constantly during the production process (Fig. 3) and similar results have been reported by Ferrer and Arboix (1986). On the other hand, Nagy et al. (1989) found that the peroxide number of Hungarian Salami decreased in the first 10 days of ripening. The final peroxide number of the fat from Sremska sausage in this trail was 9.08 meq O_2/kg fat, which is in accordance with values stated by Salgado et al. (2005) for *Chorizo de cebolla*, a Spanish traditional sausage.

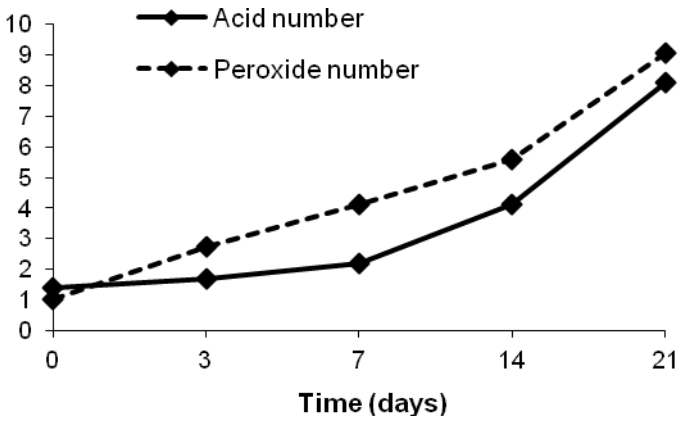

Figure 3. Changes in the fat acid number (mg KOH/g fat) and peroxide number (meq O_2/kg fat) during ripening

Changes of main chemical parameters of Sremska sausage during ripening are characterized by known tendency of increase of dry matter content (Živković et al., .2011). The proximate composition of Sremska sausage during the ripening is shown in Table 1. As a consequence of drying, at the end of ripening, sausages were characterized by a high fat content (36.42%) and low moisture content (30.21%). Ash and protein content also significantly increased during the production process, which is typical for dry fermented products (Kozačinski et al., 2008).

Table 1. Changes in proximate composition (%) of the Sremska sausage during ripening (mean ± standard deviation)

Day	Moisture	Lipid	Protein	Ash
0	58.13 ± 1.81[a]	21.23 ± 0.56[a]	17.73 ± 1.12[a]	2.85 ± 0.10[a]
3rd	56.93 ± 2.43[b]	21.69 ± 1.38[a]	18.37 ± 0.76[a]	3.04 ± 0.06[a]
7th	49.22 ± 1.06[c]	24.50 ± 0.82[b]	21.79 ± 0.93[b]	4.41 ± 0.12[b]
14th	38.45 ± 2.21[d]	31.98 ± 0.30[c]	24.39 ± 1.07[c]	5.31 ± 0.25[c]
21st	30.21 ± 0.95[e]	36.42 ± 2.09[d]	27.79 ± 1.14[d]	5.67 ± 0.18[c]

[a,b,c,d,e] Mean values in the same column with the different superscripts differ significantly ($p<0.05$)

Sensory characteristics of fermented sausages are related to breakdown products of lipids which, in turn, transform to flavour compounds during ripening and they are influenced by the quality and type of raw material, smoking, fermentation rate and time, and the rate and extent of drying (Berdagué et al., 1993; Johansson et al., 1994). Sensory evaluation of sausages at the end of production process (first day) and after 60 and 120 days of storage are presented in Figure 4. At the end of production process, final product had distinctive spicy flavor, dark red color and firm consistency.

The overall sensory quality of the sausages reduced during storage, especially in sensory scores for texture and smell, but these scores do not indicate that the product is unacceptable and they were still high (greater than 4.1). On the first and the 60th day from the production, the color was evaluated with 4.5 and after 120 days, color was evaluated with 4.3. Less average evaluation for the color was due to changes in the color after cutting (appearance of the grayish color).

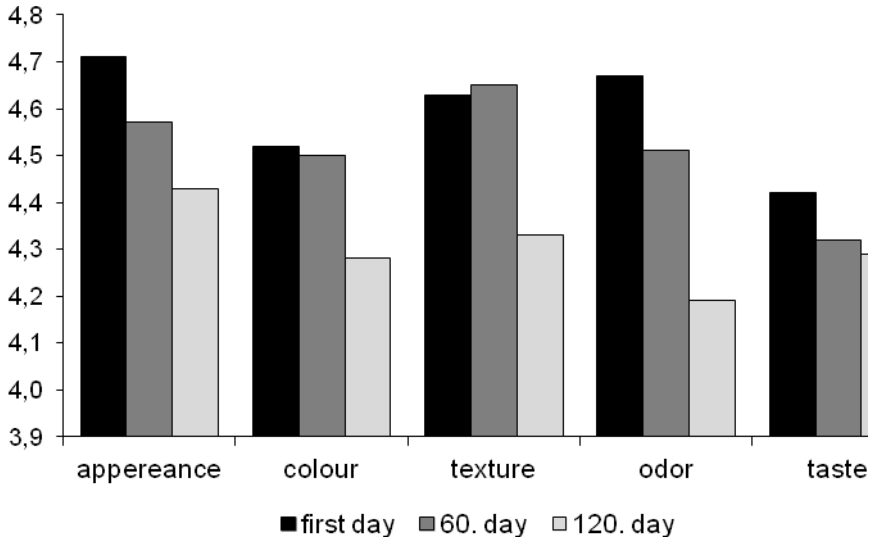

Figure 4. Sensory evaluation of Sremska sausage

CONCLUSIONS

The sausages were characterized by a long ripening period of 21 day, which is considered typical for the production in traditional smoking house. The minimum pH (4.97) was reached on 14th day, then it increased slightly to 5.18 (21st day). The values of acidity and peroxide number of the fat increased during the production process. As a consequence of drying and large weight loss, at the end of ripening, sausages were characterized by a low moisture content and high fat, protein and ash content. At the end, final product had distinctive spicy flavor, dark red color and firm consistency. During storage, overall sensory quality has slightly reduced, but sensory scores were still high for this type of product.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR31081, financed by the Ministry of Education and Science of the Republic of Serbia.

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SAFETY AND QUALITY OF TRADITIONAL CROATIAN PRODUCT „MEAT FROM TIBLICA“

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ABSTRACT:“Meat from tiblica” produced in a household and stored in controlled conditions (8°C) was sampled and examined once a month during the period of nine months. Microbial population in meat and chopped back fat during storage consisted of aerobic mesophilic bacteria, lipolytic bacteria, yeasts and molds, coagulase-negative cocci and lactic acid bacteria, without the presence of pathogens. Due to specific production (smoking and baking of meat after brining), the meat contained a lower microbial count in comparison to chopped back fat for 1 to 3 log, despite the lower a_w. Physico–chemical parameters indicate to growth of proteolytic and lipolytic activity of determined microbial groups, but not to spoilage. The grades of sensory traits of the product were rising until the seventh month of storage and even after the last month of storage, the grade was higher in comparison to the first month of storage. The results are in favor of the fact that “meat from tiblica” is a safe product of high quality.
Keywords: *“meat from tiblica”, microflora, safety, quality*

INTRODUCTION

Traditional products are characterized by specific features which are the result of production procedures and the climate conditions. The question of producing safe regional specialties outside the industry and craft, in family farms, catering and rural household is always actual in practice (Kozačinski et al., 2008). “Meat from tiblica” is a traditional product of special value and quality and it is a constituent part of cultural heritage of the area of Međimurje, North Croatia. Quality and health safety of the product depends on many factors – from quality of raw material, through technological process of production, to microbiological and chemical changes during storage.

MATERIAL AND METHODS

“Meat from tiblica” was produced in a household according to the procedure described in Figure 1. The product was stored at 8°C in controlled conditions during the period of nine months, and it was sampled and analyzed once a month.
Aerobic mesophilic bacteria, lactic acid bacteria (LAB), coagulase-negative cocci, *S. aureus*, *E. coli,* yeasts and molds, lipolytic bacteria, sulphite-reducing clostridia, enterococci *Salmonella* spp. and *L. monocytogenes* were isolated and identified in microbiological analyses of the product during storage. Standard culture-based microbiological methods were used for isolation and determination of listed microorganisms, while determination was conducted by the API system (BioMerieux, France).
Physico-chemical analyses included a determination of water activity (a_w meter, Rotronic, Switzerland), pH value (pH meter, WTW, Germany) and ammonia in meat (microdiffusion procedure by Schmidt, Živković, 1986), as well as titratable acidity (EN ISO 660:2009) and peroxide number of chopped back fat. The fatty-acid composition of the product was determined by gas chromatography. Samples were prepared according to the norm HRN EN ISO 5509:2004 and analyzed according to HRN EN ISO 5508:1999. GC analysis was

performed on CP-3800 (Varian, Palo Alto, USA) by split/splitless injector and a flame ionization detector. Capillary column DB 225ms 30m x 0.25mm was used, with 0.25 μm film thickness and a temperature program which was rising from 60°C, 4°C/min until it reached the temperature of 210°C which was maintained for 15 minutes. Helium was used as a carrier gas (mobile phase) at a flow rate of 1mL/min. The injector temperature was 250°C and sample split ratio was 1:30, whereas a flame ionization detector temperature was 250°C. Samples were injected manually (0.1 μL).
Five evaluators participated in the sensory evaluation of "meat from tiblica". Each sensory indicator was evaluated according to a scoring system 1 to 5. Sensory analysis of a finished product was conducted according to a shortened protocol by Cocolin et al. (2005) which included the grade for color, odor and consistency of meat and chopped back fat, as well as the grade for cross-section appearance and taste of meat.

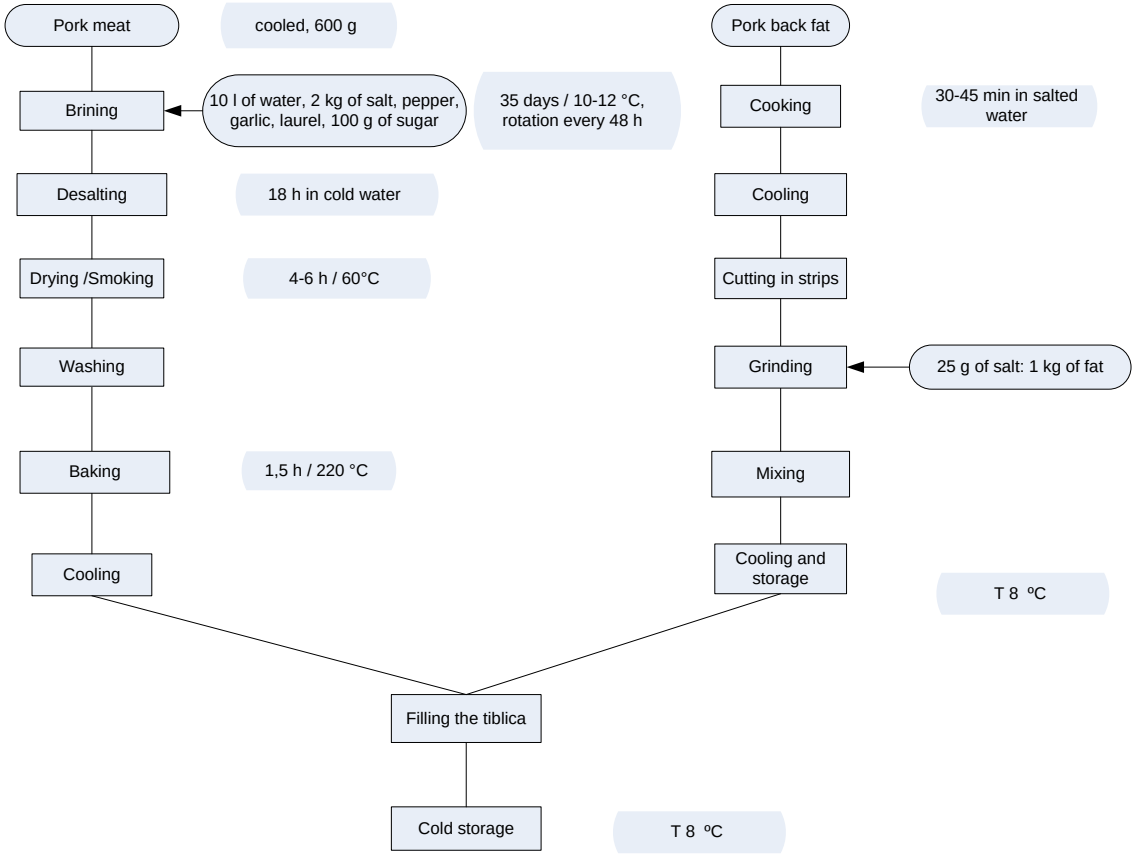

Figure 1. Procedure of "meat from tiblica" production

RESULTS AND DISCUSSION

Results of monitoring of selected microorganisms in "meat from tiblica" are presented in Table 1. Compared to initial number, after 9 months of storage we find slight decrease of coagulase-negative cocci and aerobic mesophilic count (0.4-0.5 logs) and significant increase of lipolitic bacteria and yeast/moulds (1 and 1.3 logs, respectively). After nine months of storage, In product's chopped back fat, the number of yeasts and molds, aerobic mesophilic bacteria, lipolytic bacteria and coagulase-negative cocci were within a narrow range from 5.15 to 5.62 $\log_{10}$ CFU/g. The LAB number was lower during storage in meat (2-3 $\log_{10}$ CFU/g) than in chopped back fat (4-5 $\log_{10}$ CFU/g). It is important to emphasize that LAB were found in the meat after second month of storage even though the meat was baked, so it can be presumed that they were growing up to a certain point in meat too, as a result of an interaction of meat with chopped back fat. Our finding confirms the results of other authors who proved that *Micrococcaceae,* LAB, then yeasts and molds have a significant

part in maturating process of meat products (Samelis et al., 1998, Lücke, 2000, Zdolec, 2007, Zdolec et al., 2007, Martín et al., 2008). LAB were not a dominant population in our product as it is the case in fermentation of sausages, but we can assume that they do affect sensory characteristics of a finished product, along with other factors of maturating and storage.

Bacteria *Salmonella* spp. and *L. monocytogenes,* then enterobacteria, sulphite-reducing clostridia, enterococci, *E. coli* and *S. aureus* were not determined (or they were below the limit of detection) in any sample of meat and chopped back fat during storage of "meat from tiblica", which is important to be emphasized regarding product's safety.

Table 1. Microbial count (log_{10} CFU/g) of "meat from tiblica" during storage

Storage period, month	Aerobic mesophilic bacteria	Yeasts and molds	Lipolytic bacteria	Coagulase-negative cocci	Lactic acid bacteria
Meat					
1	3.69±0.06	2.79±0.29	2.72±0.03	3.53±0.08	<2*
2	3.62±0.03	2.42±0.20	3.11±0.16	3.60±0.03	2.10±0.17
3	3.57±0.04	3.56±0.06	3.63±0.03	3.85±0.01	2.37±0.21
4	3.94±0.01	4.32±0.03	4.00±0.00	3.62±0.03	3.37±0.12
5	3.82±0.03	4.38±0.05	3.93±0.03	3.90±0.01	3.10±0.17
6	3.84±0.03	3.95±0.02	3.29±0.13	3.25±0.05	3.00±0.13
7	3.64±0.04	4.02±0.05	3.93±0.03	3.23±0.04	3.02±0.07
8	3.45±0.04	4.23±0.03	3.12±0.08	3.23±0.04	2.31±0.27
9	3.25±0.05	4.11±0.16	3.77±0.01	3.04±0.09	2.20±0.17
Chopped back fat (cooked and minced)					
1	6.30±0.27	4.38±0.08	5.35±0.31	5.40±0.49	4.00±0.13
2	6.38±0.06	4.53±0.08	5.94±0.01	5.19±0.15	4.68±0.06
3	6.75±0.04	4.36±0.07	6.16±0.14	5.59±0.03	4.81±0.18(
4	6.26±0.04	4.49±0.07	6.19±0.03	5.57±0.01	4.85±0.10
5	6.34±0.02	4.57±0.01	6.33±0.03	5.88±0.02	4.93±0.03
6	6.36±0.07	4.33±0.08	6.60±0.01	5.93±0.03	4.90±0.06
7	6.43±0.02	4.98±0.03	6.76±0.05	6.34±0.02	5.01±0.06
8	6.26±0.04	5.40±0.06	6.21±0.21	5.97±0.03	4.75±0.04
9	5.62±0.06	5.05±0.16	5.47±0.02[a]	5.15±0.04	4.35±0.05

The pH value of meat was decreasing mildly and continuously until the sixth month of storage (0.4 units) and then slightly increased to final value of 6.22 (Table 2.). The similar pH trend was determined in chopped back fat (Table 2.). The a_w results in meat samples show that values remained at the approximately same level during storage, whereas they were growing in chopped back fat, (Table 2.). The ammonia content in the meat was growing continuously during storage (Table 2.). Peroxide number and titratable acidity in chopped back fat were also growing continuously (Table 3.). If we interpreted the results according to Živković (1986), who listed that sensory flawless fat should have titratable acidity lower than 2 and peroxide number lower than 3, the sample would not be considered sensory flawless in the last month of storage because its titratable acidity was 4.11. But, fat and chopped back fat are not equal products; fat is obtained by melting (high temperatures), and chopped back fat by cooking and then mincing back fat tissue.

Table 2. A_w, pH and NH_3 values during the storage of "meat from tiblica"

Storage period, month	a_w of meat	a_w of chopped back fat	pH of meat	pH of chopped back fat	NH3 in meat (mg%)
1	0.887±0.00 B	0.800±0.00	6.35±0.01	6.80±0.02	1.02±0.17
2	0.881±0.00 L	0.812±0.00	6.29±0.01	6.49±0.01	1.53±0.17
3	0.867±0.00 B	0.829±0.00	6.15±0.01	6.47±0.01	2.04±0.17
4	0.885±0.00 L	0.848±0.00	6.11±0.01	6.37±0.01	2.61±0.26
5	0.880±0.00 N	0.860±0.00	6.08±0.01	6.43±0.02	3.40±0.17
6	0.874±0.00 Lo	0.849±0.00	5.95±0.01	6.52±0.01	4.31±0.26
7	0.877±0.00 Lo	0.851±0.00	6.06±0.03	6.59±0.01	5.27±0.17
8	0.883±0.00 S	0.849±0.00	6.18±0.01	6.59±0.02	6.40±0.26
9	0.884±0.00 S	0.848±0.00	6.22±0.01	6.63±0.01	8.56±0.35

L-leg; Lo- Loin; S- shoulder; B- belly; N- neck.

Table 3. Titratable acidity and peroxide number during storage of „meat from tiblica"

Storage period, month	Titratable acidity	Peroxide number
1	1.05±0.07	0.14±0.02
2	1.18±0.06	0.4±0.01
3	1.28±0.03	0.51±0.01
4	1.48±0.05	0.56±0.01
5	1.56±0.06	0.66±0.01
6	1.76±0.05	0.74±0.01
7	2.28±0.06	0.83±0.00
8	2.98±0.06	0.97±0.01
9	4.11±0.03	1.24±0.02

Table 4. Results of sensorial evaluation of "meat from tiblica"

Storage period, month	1	2	3	4	5	6	7	8	9
Total - meat	4.16	4.2	4.4	4.32	4.4	4.54	4.76	4.64	4.6
Total - chopped back fat	4.4	4.6	4.87	4.87	4.87	4.93	4.87	4.87	4.87
Total impression	**4.28**	**4.4**	**4.63**	**4.59**	**4.63**	**4.74**	**4.81**	**4.75**	**4.73**

We determined an increase in the concentration of SFA in the product, and a decrease in MUFA and PUFA concentrations during storage (Table 5.). The same trend has been shown by the results of fatty acid composition of different kinds of cooled or frozen meat (Kesava and Kowale, 1993; Lee et al., 1994; Hernandez et al., 1999; Nürnberg et al., 2006; Alvarez et al., 2009; Mahecha et al., 2009; Sučić, 2010). Oleic, palmitic, stearic and linoleic acids were the most frequently presented fatty acids in meat and chopped back fat in our research, which is in accordance with the results of Njari (1986) and Valsta et al. (2005). A favorable PUFA/SFA ratio has also been confirmed (Wood et al., 2003).

The samples in our research were stored in controlled conditions at 8°C. Such storage is in accordance with the recommendation of Renčelj (1997) and Kolakowska et al. (2003) that the appearance of an unpleasant aroma and oxidation can be prevented or slowed down by using dark or non-transparent packaging and cold storage. The samples of the finished product met the requirements for sensory characteristics of "meat from tiblica" listed by Renčelj (1997). The meat got softer and juicier with the duration of the storage, so the grades for sensory characteristics were consequently increasing until the seventh month of storage. Particularly good grades were given to characteristics of odor and taste, then meat consistency. When the storage was completed, the total grade for meat and chopped back fat was 4.74, which is higher for 0.43 than after the first month of storage (Table 4.). A full,

mature (more aromatic) taste achieved and kept by our product until the end of storage, as well as softer meat, can be attributed to interaction, i.e. changes, of muscle and fatty tissue, the same as with Renčelj (1997).

Table 5. Fatty-acid composition of meat and back fat of "meat from tiblica" during storage

	Storage period, month								
Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Meat									
C12:0	0.2	0.2	0.1	0.1	0.1	0	0	0	0
C14:0	1.4	1.5	1.5	1.4	1.4	1.8	1.9	1.3	1.5
C16:0	25.2	25.4	25.7	25.8	26	26.9	27.4	28.2	28.5
C17:0	0.3	0.4	0.4	0.4	0.4	0.5	0.3	0.4	0.4
C18:0	11.9	11.8	11.9	12.3	12.3	12	12.3	13	13.1
C20:0	0.2	0.3	0.2	0.2	0.2	0	0.1	0.1	0.1
∑SFA	**39.2**	**39.6**	**39.8**	**40.2**	**40.4**	**41.2**	**42**	**43**	**43.6**
C16:1	2,9	2.9	2.5	2.5	2.7	2.8	2.8	2.7	2.5
C17:1	0	0	0	0.1	0	0	0	0	0
C18:1	48.5	48.3	48.6	48.3	48.2	47.7	47.2	47	47
C20:1	0.9	1	1	0.9	0.9	0.7	0.8	0.7	0.7
C22:1	0.1	0.1	0	0	0	0	0	0	0
∑MUFA	**52.4**	**52.3**	**52.1**	**51.8**	**51.8**	**51.2**	**50.8**	**50.4**	**50.2**
C18:2	7.8	7.3	7.3	7.4	7.2	7.6	6.9	6.4	6.1
C20:2	0.3	0.4	0.4	0.3	0.3	0	0.2	0.1	0.1
C18:3	0.3	0.4	0.4	0.3	0.3	0	0.1	0.1	0
∑PUFA	**8.4**	**8.1**	**8.1**	**8**	**7.8**	**7.6**	**7.2**	**6.6**	**6.2**
Chopped back fat (cooked and minced)									
C12:0	0	0.1	0	0.1	0.1	0.1	0	0.1	0.1
C14:0	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.5	1.6
C16:0	24.6	24.7	25.4	25.2	25.1	25.7	25.8	25.8	26
C17:0	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4
C18:0	12.3	12.4	12.3	12.4	12.7	12.8	12.9	13.3	13.5
C20:0	0.1	0.2	0.3	0.3	0.3	0.2	0.3	0.2	0.3
∑SFA	**38.8**	**39.2**	**39.7**	**39.8**	**40**	**40.6**	**40.8**	**41.3**	**41.9**
C16:1	2.2	2.2	2.1	2.2	2.2	2	2.1	2.2	2
C17:1	0	0	0	0	0	0,4	0	0,1	0
C18:1	48.2	48.3	48.2	48.2	48	47.8	48	47.5	47.2
C20:1	1.2	1.2	1.1	1	1.1	1.1	1.2	1.1	1.2
C22:1	0	0	0	0	0	0,1	0	0	0,1
∑MUFA	**51.6**	**51.7**	**51.4**	**51.4**	**51.3**	**51.4**	**51.3**	**50.9**	**50.5**
C18:2	8.8	8.2	8.2	8	7.9	7.3	7.3	7.1	7.2
C20:2	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.4	0.3
C18:3	0.3	0.4	0.3	0.4	0.4	0.3	0.2	0.3	0.1
∑PUFA	**9.6**	**9.1**	**8.9**	**8.8**	**8.7**	**8**	**7.9**	**7.8**	**7.6**

SFA= Saturated Fatty Acid; MUFA = Monounsaturated Fatty Acid; PUFA = Polyunsaturated Fatty Acid

CONCLUSIONS

Characteristcs of „meat from tiblica", especially gustatory quality, depend on the interaction of meat and chopped back fat during storage of product. The product received high grades for sensory characteristics during nine months of storage and met the demands in terms of microbiological quality and safety of meat products. Microbial population consisted of aerobic mesophilic and lipolytic bacteria, yeasts and molds, coagulase-negative cocci, and LAB to a lesser extent, without the presence of pathogens. Physico-chemical changes of product during storage indicate to a growing lipolytic and proteolytic enzyme activity in determined groups of microorganisms and probably tissue enzymes, which possibly contributed to sensorial quality of "meat from tiblica". The product was sustainable during nine months at 8°C.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project Microbiological quality and shelf life of foodstuffs of animal origin (053-0531854-1853), financed by the Ministry of Science, Education and Sports, the Republic of Croatia.

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POSSIBILITY OF USING POTASSIUM CHLORIDE AND GLYCINE AS A SUBSTITUTES FOR SODIUM CHLORIDE IN HOMEMADE SLAVONIAN SAUSAGES

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ABSTRACT: The excessive intake of sodium (Na) has been linked to hypertension and consequently to the increased risk of stroke and premature death from cardiovascular diseases. The reduction of added NaCl in fermented meat products has been proposed in order to decrease the amount of sodium in the diet. The effect of partial replacement of NaCl by KCl and glycine to physicochemical, microbiological and sensory characteristics of homemade Slavonian sausages was analyzed. According to the results, the partial substitution of NaCl by KCl and glycine had no effect on basic composition, pH, a_w, color coordinates and texture of the fermented sausage. The reduction of NaCl slightly increased the number of *Enterobacteriaceae* and *Staphylococcus aureus*, but all samples were still microbiologically safe, according to the EU legislation. According to the sensory scoring test the highest rated sausage was the one containing 1% NaCl, 0.9% KCl and 0.1% glycine, the second rated was the control sausage with 2% NaCl and the third one was the sausage with 1% NaCl and 1% KCl. According to these results, it is possible to achieve the reduction of 50% of NaCl in homemade Slavonian sausages by replacing it with KCl (substitution level between 30 and 50%), and glycine (substitution level between 10 and 20%) while still maintaining traditionally desirable sensory properties.
Key words: *homemade Slavonian sausage, lowering level of NaCl, partially replacing NaCl, the effect on physicochemical, microbiological and sensory characteristics*

INTRODUCTION

Reduction of NaCl content became important measure in improvement of health, because eexcessive intake of sodium (Na) has been linked to hypertension and consequently to the increased risk of stroke and premature death from cardiovascular diseases. The main source of sodium (Na) in the diet is sodium chloride (NaCl) (Ruusunen & Puolanne, 2005). Meat and meat products contribute 20.5% to the sodium intake in Ireland, 20.8% to the sodium intake in UK, and 21.0% to the sodium intake in USA (Desmond, 2006). Salt (sodium chloride, NaCl) is an essential ingredient in dry fermented sausages, and therefore they are one of the biggest sources of dietary NaCl from meat products (3.3 – 4.8 g/100g final product). NaCl in these products contributes to water-holding capacity, fat binding, color, flavor and texture. Salt also plays an important role in ensuring the microbial stability of dry fermented sausages (Gelabert, Gou, Guerrero, & Arnau, 2003; Zanardi, Ghidini, Conter, & Ianieri, 2010). In October 2003 the FSA announced a “salt model” in order for the UK food industry to reduce the amount of salt in food products. In August 2005 the FSA proposed new target (g/100 g) which was supposed to be achieved by 2010 suggesting a maximum of 1.4 g of salt/550 mg of sodium for sausages (Desmond, 2006; Food Standard Agency [FSA], 2006).
Apart from lowering the level of salt added to the products, there are currently three major approaches to reduce the salt content in processed foods: the use of salt substitutes (KCl,

phosphates, potassium lactate, glycine, calcium ascorbate, calcium chloride); the use of flavor enhancers and masking agents; the optimization of the physical form of salt (Desmond, 2006). Most authors studied the effect of partial replacement of sodium chloride by KCl, potassium lactate (K-lactate), glycine, calcium ascorbate, calcium chloride (Gou, Guerrero, Gelabert, & Arnau, 1996; Gimeno, Astiasaran, & Bello, 1998; Gimeno, Astiasaran, & Bello, 1999; Gimeno, Astiasarán, & Bello, 2001; Ibanez et al., 1995; Gelabert et al., 2003; Guardia, Guerrero, Gelabert, Gou, & Arnau, 2006; Guardia, Guerrero, Gelabert, Gou, & Arnau, 2008). A salt substitution level of 40% by KCl (Gelabert et al., 2003; Askar, Elsamahy, Shehata, & Tawfik, 1993; Gou et al., 1996), and of 30% with K-lactate and 20% with glycine affects unfavorable flavor and textural changes (Gelabert et al., 2003). Microbial stability of sausages produced by partial substitution of NaCl by KCl, K-lactate and glycine was slightly reduced (Gelabert et al., 2003).

There is no accurate data on sodium intake from meat products in Croatia and no research article on reducing salt in the Croatian autochthonous meat products has been published yet. Therefore, the aim of this research was to explore possibility of partially replacing NaCl with KCl and glycine in the homemade Slavonian sausages, in accordance to microbiological, physicochemical and sensory characteristics and consumer's preference.

MATERIALS AND METHODS

Sausage preparation

A homemade Slavonian sausage is a type of smoked, fermented and dried-ripened sausage produced in Slavonia (Eastern region of Croatia), made from pig meat of 12 months old crossbreed between Swedish Landrace and Large White, raised on the family farm and slaughtered in a commercial slaughterhouse. Pig carcasses were transported to a small scale facility in Eastern Croatia where the sausages were produced.

The meat from the third quality parts of the pig such as pork belly and *cca.* 20% of the second quality parts, such as pork shoulder, was cut into stripes 30 cm long, 10 cm wide and 3 cm thick and placed in a freezer until the temperature of the meat reaches -2 to -5 °C. The meat is than grinded through a grinding plate with holes of 7 mm in diameter. Six different batches of homemade Slavonian sausages, (control batch with traditional formulation - 2.0% of NaCl) and five batches with the partial substitution of NaCl by KCl and glycine (Table 1) were manufactured. All six batches (5 sausage samples of each batch) were made with a standard formulation for homemade Slavonian sausage: raw pork of third and second quality (96.8%), garlic (0.2%), red hot paprika powder (0.4%) and sweat paprika powder (0.6%) with variable NaCl, KCl and glycine content (Table 1).

Table 1 Percentage of reduction and molar substitution of NaCl by KCl and glycine

	Control sample	$SB_{50;50}$	$SB_{60;40}$	$SB_{70;30}$	$SB_{50;40;10}$	$SB_{50;30;20}$
NaCl	100	50	60	70	50	50
KCl	-	50	40	30	40	30
Glycine	-	-	-	-	10	20

Control sample: 20 g NaCl/kg product.
SB – sausage batch

The mix was stuffed into natural casing (pig's thin intestine (lat. *intestinum tenue*)) and the resulting sausages were smoked with dry hard wood every second day for two weeks. The temperature and relative humidity at this stage were about 18 to 20 ºC and 70 to 90% respectively. After smoking, the

Slavonian homemade sausages were left for the ripening process in a dark room with the temperature from about 14 to 17 ºC and relative humidity 70 to 80% for 45 days. The sausages were made in a non-industrial environment, characterized by a small-scale batch production with a limited degree of mechanization, using traditional techniques and strongly defined by the climate and region of origin. Once collected, the samples were placed in a

portable cooler and transported to the laboratory within one hour, and then stored in a refrigerator (below 4°C) up to maximum 24 h before analysis.

Physicochemical parameters

Before physicochemical analysis sausage samples were tripartite and, after removing the outer casing from each sausage, the edible part was ground until a homogenous mass was obtained.

The FoodScan Meat Analyser was used to determine moisture, total protein, total fat and collagen according to the Association of Official Analytical Chemists method (AOAC, 2007. 04.)

pH values were determined in a homogenate of the sample with distilled water (1:10, p/v) using pH/Ion 510 – Bench pH/Ion/mV Meter (Eutech Instruments Pte Ltd/ Oakton Instruments, USA), according to the ISO 2917:1999 Method (ISO, 1999) and pH/Ion 510 Instruction Manual.

Salt content (sodium chloride (NaCl)) was determined according to the ISO 1841:1970 Method (ISO, 1970).

Water activity (a_w) was determined using a Rotronic Hygrolab 3 (Rotronic AG, Bassersdorf, Switzerland) at room temperature (20 ± 2 ºC).

Five sausage sample of each batch were analyzed. Three determinations for each parameter were measured in each sample. Results shown in the Table 3 are the means of the five sausage samples.

Determination of color

Samples for instrumental color measurement were cut in 1 cm thick slices after removing the outer casing. Color measurements (L*, a*, and b* values) were taken using a Hunter-Lab Mini ScanXE (A60-1010-615 Model Colorimeter, Hunter-Lab, Reston, VA, USA). The instrument was standardized each time with a white and black ceramic plate (L_0 = 93.01, a_0 = -1.11, and b_0 = 1.30). The Hunter L*, a*, and b* values respectively correspond the lightness, greenness (-a*) or redness (+a*), and blueness (-b*) or yellowness (+b*). The color measurements were performed on homemade Slavonian sausage at room temperature (20 ± 2 ºC). Five sausage sample of each batch were analyzed. The color of each sample was measured ten times. Results shown in the Table 4 are the means of the five sausage samples.

Texture profile analysis

A Universal TA-XT2i texture analyzer was used to conduct texture profile analysis (Bourne, 1978). Samples for texture analysis (TPA) were cut in 1×1×1 cm cubic samples after removing the outer casing. Five sausage sample of each batch were analyzed. Ten cubic samples 1×1×1 cm of each sausage sample were compressed twice to 60% of their original height with a compression platen of 75 mm in diameter. Textural analyses were performed at ambient temperature. Force-time deformation curves were recorded at a crosshead speed of 5 mm/s and recording speed also 5 mm/s. Hardness, springiness, cohesiveness, gumminess and chewiness were evaluated. These parameters were obtained using the Texture Expert for Windows (version 1.0) Stable Micro Systems. Results shown in the Table 5 are the means of the five sausage samples.

Sensory analysis

Scoring test

A group of 5 selected and trained sensory panelists (ASTM, 1981; ISO 8586-1, 1993 and ISO 8586-2, 1994) evaluated the sensory characteristics of the sausages studied. Appearance, texture, taste, color and odor were assessed using the scoring test in which samples were given scores from 1 (very poor) to 5 (excellent). The overall sensory quality takes into account all factors of sensory quality that need to be evaluated by sensory panelists, was calculated according to the following formula: overall sensory quality = (appearance × 2) + (texture × 6) + (taste × 8) + (color × 2) + (odor × 2). This formula is based

on the relative importance of the different sensory characteristics which were calculated according to the opinion of 5 experts asked to assess, in an organized open discussion, those different sensory characteristics for this specific experiment.

Microbiological analysis

Microbiological analyses were performed on the 5 samples of each type of sausage. Classical microbiological methods were used for isolation and identification of the microbial population. *Salmonella* was analyzed by Method ISO 6579 (ISO, 2003a); *Enterobacteriaceae* by Method ISO 5552 (ISO, 1997); *Staphylococcus aureus* by Method ISO 6888-1 (ISO 1999); *Sulphite-reducing Clostridia* by Method 15213 (ISO, 2003b) and *Listeria monocytogenes* by Method ISO 11290-1/A1 (ISO, 2004).

Data analysis

Differences among the average values of the same physicochemical, color and texture parameters between sausage batches with different NaCl content were analyzed through the analysis of variance (ANOVA). Differences between scores for sensory parameters between sausage batches were also analyzed through the analysis of variance (ANOVA) and Fisher's least significant difference test (LSD), with significance defined at $P<0.05$. For determination which sausage samples differ one from another according to mean score values for every sensory parameter and overall sensory quality, Friedman's nonparametric ANOVA was used. Moisture content, fat content, protein content, collagen content, NaCl content, pH, $a_{w,}$ color parameters, textural parameters and sensory characteristics were subjected to correlation analysis (multivariate method) to determine the possible statistical relationships between them. Statistical analysis was carried out Statistica ver. 7.0 StatSoft Inc. Tulsa, OK. USA.

RESULTS AND DISCUSSION

Table 2 shows basic composition, pH and a_w of 6 different batches of homemade Slavonian sausage and raw meat used for sausages preparation. All sausage mixtures were prepared from the same raw meat mixture and the only difference between batch formulations was the NaCl, KCl and glycine content. Initial values of pH, a_w and moisture content were very similar for all batches (almost the same as in raw meat).

Table 2 Basic composition, pH, a_w and microbiology of 6 different batches of homemade Slavonian sausage and raw meat

Parameter	Control sample	$SB_{50;50}$	$SB_{60;40}$	$SB_{70;30}$	$SB_{50;40;10}$	$SB_{50;30;20}$	Raw meat
Moisture (%)	32.23^{a}	31.05^{b}	28.85^{d}	28.48^{d}	29.50^{c}	28.76^{d}	72.97
Protein (%)	31.79^{e}	33.24^{c}	33.87^{b}	32.91^{d}	33.97^{b}	37.17^{a}	21.59
Fat (%)	26.18^{e}	27.12^{d}	27.45^{c}	28.93^{b}	29.10^{a}	28.96^{b}	4.96
Collagen (%)	4.32^{bc}	4.84^{abc}	4.90^{abc}	3.94^{c}	5.06^{ab}	5.41^{a}	1.31
NaCl (%)	3.86^{a}	1.94^{d}	2.01^{c}	2.68^{b}	1.89^{e}	1.95^{d}	0.11
a_w	0.89^{a}	0.88^{a}	0.86^{c}	0.85^{d}	0.87^{b}	0.86^{c}	1.00
pH	5.29^{a}	5.24^{b}	5.15^{d}	5.18^{c}	5.25^{b}	5.17^{cd}	5.48
Staphylococcus aureus (log_{10} CFU/g)	-	1.70*	2.00*	2.00*	2.00*	2.00*	2.17*
Enterobacteriaceae (log_{10} CFU/g)	-	1.60*	1.30*	1.30*	1.48*	1.00**	2.90**

^{a,b,c,d,e} Within a row, least squares means with different superscripts differ significantly ($p<0.05$)

*Value appears in one of the 5 samples analyzed for each sausage batch;

** Value appears in two of the 5 samples analyzed for each sausage batch

Although all batches followed the same drying process, the highest moisture content and pH value were observed in the control sausage batch (sausage with the highest NaCl content).

Except that, no clear effect (no statistically significant correlation between tested parameters and NaCl content) was found in the basic composition, pH and a_w, which could be the consequence of the reduction and partial replacement of NaCl. There is a statistically significant positive correlation between protein and glycine content (0.90), moisture content and a_w (0.94), moisture content and pH (0.86), pH and a_w (0.81) which are expected and statistically significant negative correlation between fat and moisture content (-0.83).
Table 3 shows the results of instrumental color measurement of 6 different batches of homemade Slavonian sausage. The control sausage batch shows the highest L* (lightness) value. That is in contrast to the findings reported by Zanardi et al. (2010) and Gimeno et al. (1999), but they used a mixture of NaCl, KCl and $CaCl_2$, and particularly $CaCl_2$ could be the possible reason for the product with higher L* values. There is also a positive significant correlation between moisture content, and L* value (0.95) which isn't in agreement with Pérez-Alvarez, Fernández-López, Sayas-Barberá, and Cartagena-Graciá (1998). There is no statistically significant correlation between color coordinates and NaCl content, indicating that salt reduction or partial replacement with KCl and glycine doesn't affect sausage color.

Table 3 Instrumental color measurement of 6 different batches of homemade Slavonian sausage

Color characteristics	Control sample	$SB_{50;50}$	$SB_{60;40}$	$SB_{70;30}$	$SB_{50;40;10}$	$SB_{50;30;20}$
L*	32.61^{a}	31.62^{ab}	30.32^{c}	30.37^{c}	30.44^{bc}	30.79^{bc}
a*	20.77^{b}	21.80^{ab}	20.86^{b}	21.56^{b}	20.97^{b}	22.77^{a}
b*	14.01^{b}	14.68^{ab}	13.93^{b}	14.90^{ab}	14.05^{b}	15.59^{a}

^{a,b,c} Within a row, least squares means with different superscripts d

Textural properties are given in Table 4. Control sausage showed the lowest value of hardness and gumminess and the highest value of springiness, but otherwise, no other clear effect was found in the texture as a consequence of NaCl reduction. Sausage batches with higher moisture content and a_w are less hard (negative significant correlation between moisture content and hardness (-0.82) and a_w and hardness (-0.91)). Sausage batches with higher pH are more springy (positive significant correlation between pH and springiness (-0.98) (Tables 2, 4).

Table 4 Texture profile analysis (TPA) of 6 different batches of homemade Slavonian sausage

Parameter	Control sample	$SB_{50;50}$	$SB_{60;40}$	$SB_{70;30}$	$SB_{50;40;10}$	$SB_{50;30;20}$
Hardness (g)	2889.65^{d}	3049.80^{cd}	4554.38^{ab}	4830.71^{a}	4092.65^{b}	3458.13^{c}
Springiness (mm)	0.52^{a}	0.45^{bc}	0.43^{c}	0.44^{c}	0.49^{ab}	0.45^{bc}
Cohesiveness	0.36^{a}	0.34^{ab}	0.35^{ab}	0.34^{ab}	0.35^{ab}	0.32^{b}
Gumminess (g)	1027.98^{b}	1048.81^{b}	1569.74^{a}	1630.97^{a}	1449.06^{a}	1117.35^{b}
Chewiness (g x mm)	540.68^{b}	464.57^{b}	667.09^{a}	710.27^{a}	703.23^{a}	499.23^{b}

Mean values (with standard deviation and coefficient of variation in parentheses)
^{a,b,c,d} Within a row, least squares means with different superscripts differ significantly ($p<0.05$)

Table 5 shows sensory scores for appearance, texture, taste, color and odor of 6 different batches of homemade Slavonian sausage, which were given by 5 trained sensory panelists using a scoring test. Scores indicate that there is no significant difference between samples in texture, taste, color and odor, while a small but significant difference in appearance was observed (Fisher's LSD test). The overall sensory quality was calculated according to the following formula: overall sensory quality = (appearance × 2) + (texture × 6) + (taste × 8) + (color × 2) + (odor × 2), taking into account the relative importance of the different sensory characteristics. The sausage with 1% NaCl, 0.9% KCl and 0.1% glycine was highly scored for overall sensory quality. The second rated was the control sausage with 2% NaCl and the third rated was the sausage with 1% NaCl and 1% KCl, but there was no statistically significant difference between sausage batches in overall sensory quality scores (according

to Friedman's test results). That indicates that partial replacement of NaCl with KCl and mixture of KCl and glycine weren't perceived by the sensory panel.

Table 5 Results from sensory scoring test of 6 different batches of homemade Slavonian sausage

Sensory characteristics	Control sample	$SB_{50;50}$	$SB_{60;40}$	$SB_{70;30}$	$SB_{50;40;10}$	$SB_{50;30;20}$
Appearance	3.4^{ab}	3.4^{ab}	2.6^{b}	3.4^{ab}	3.6^{a}	3.0^{ab}
Texture	4.0^{a}	4.0^{a}	3.8^{a}	3.6^{a}	4.0^{a}	4.0^{a}
Taste	3.6^{a}	3.6^{a}	3.8^{a}	3.4^{a}	3.8^{a}	3.6^{a}
Color	4.2^{a}	4.2^{a}	4.0^{a}	4.0^{a}	4.0^{a}	4.2^{a}
Odor	3.8^{a}	3.8^{a}	3.4^{a}	3.4^{a}	4.4^{a}	3.8^{a}
Overall quality (Friedman's test)	76.4^{a}	75.6^{a}	74.4^{a}	70.4^{a}	78.4^{a}	74.4^{a}

^{a,ab,b} Within a row, least squares means and ranking sums with different superscripts differ significantly ($p<0.05$)

In relation to sensory analysis, there is a significant negative correlation between instrumental measured hardness and sensory score for texture (-0.83). Significant positive correlation was found between the moisture content and the sensory scores for color (0.94) and L* value and sensory scores for color (0.92). Significant negative correlation was found between fat content and sensory score for color (-0.83), which indicate that less hard sausages with higher moisture content and lightness and lower fat content would be perceived as sausages of highest quality.
Microbiological quality as well as safety, as a consequence of the NaCl reduction, was not affected according to the currently valid Croatian legislation adjusted to Commission regulation (EC) No 2073/2005 (R 2073, 2005) and (EC) No 1441/2007 (R 1441, 2007). But, in sausage batches with partially replaced salt content, in one of 5 samples analyzed for each sausage batch, slightly elevated, but below legislative limits, counts of *Staphylococcus aureus* and *Enterobacteriaceae* were detected, probably as a result of salt reduction. There is also a significant negative correlation between the total count of *Enterobacteriaceae* and NaCl content (-0.88) as well as with the total count of *Staphylococcus aureus* and NaCl content (-0.88).

CONCLUSION

Partial replacement of NaCl with KCl (30-50%) and glycine (10-20%) has no negative effect on physicochemical properties and microbiological safety of homemade Slavonian sausages. According to the sensory scoring test the highest rated sausage was the one containing 1% NaCl, 0.9% KCl and 0.1% glycine, the second-rated was the control sausage with 2% NaCl and the third-rated was the sausage with 1% NaCl and 1% KCl, but there were no statistically significant difference between sausage batches.
According to these results, it is possible to achieve the reduction of 50% of NaCl in homemade Slavonian sausages by replacing it with KCl (substitution level between 30 and 50%), and glycine (substitution level between 10 and 20%) in order to maintain traditionally desirable sensory properties

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CHARACTERISTICS OF COLOR AND TEXTURE OF FERMENTED "UZICKA" SAUSAGES PRODUCED IN THE TRADITIONAL WAY

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ABSTRACT: With the aim of preserving the quality of traditional fermented sausages and provision of continuity in production, a task of this work is to determine the quality of "užička" sausages, which is traditional Serbian product. This paper presents the results of sensory evaluation of traditional "užička" sausage and the results color and texture instrumental measurement, at the end of the production process.
Sausages were made from I category (50%) and II category (20%) beef, II category (20%) pork meat, fatty tissue (10%), nitrite curing salt (2.50%), sucrose (0.33%) and spices (1.25%). mixture was stuffed in bovine small intestines. Sausages were smoked in traditional smokehouses - smoking, fermentation, ripening and drying lasted 21 days. Sausages were made in three replicates.
Sensory properties of sausages (color, cut surface, coherence, tenderness) were evaluated using quantitative descriptive test (scale of intensity, of 1 to 10) at the end of production. Color and texture of "uzicka" sausage instrumentally ("Chromameter CR-400" and "INSTRON model 4301"), were analyzed, aiming to define the total quality.
Results of sensory avaluation showed that the most desired properties had sausages from a third fermentation, produced in the January, when climatic conditions were optimal for the production), and the results of instrumental measurements of color and texture are in agreement with the results of sensory evaluation.
Key words: *"Užička" sausage, sensory analysis, colour and texture instrumental analysis*

INTRODUCTION

Dry fermented sausages have always been appreciated and demanded by consumers for its quality – recently, they also became the subject of growing interest in research area. Available literature provides information about the authentic ways of fermented sausages production with special emphasis on microclimate conditions. Also, detailed microbiological, physico-chemical, sensory and other research are conducted in this area (Ambrosiadis et al., 2004; Morretti et al., 2004; Cocolin et al., 2005; Gasparik-Reichardt et al., 2005; Turubatović et al., 2004; Vesković-Moračanin, 2009). Flavour and other properties of traditionally fermented sausages are influenced by the selection and quality of basic raw material, metabolic activity of the present epiphytic microflora, physical and chemical changes due to drying, smoking, enzymatic breakdown of proteins and fats, external factors (temperature, relative humidity and air circulation), duration of ripening and other (Mendoza, 2001).
„Užička" (domestic) sausage and „kulen" are the most popular national representatives of this group of sausages.Traditional (household) production of these sausages takes place in colder periods of the year (November - January). Pork is the basic component, obtained from animals (often „Mangulica" breed) slaugtered on the same day of manufacture. Used meat is of high quality (mostly, shoulder, sometimes leg). In the cases when meat of lower quality is used (higher content of fat and connective tissue), it has to be grinded. Sometimes, beef can

be also added. Other ingredients are common salt, sweet or hot grinded paprika and garlic. Sausages are smoke for several days in traditional smokehouses (twice a day) and are air-dried to the end of the winter (Tojagić, 1996).
Such way of production was the only viable option in the past regarding food preservation since no other means were available at the time. Sausages had high nutritional value and desirable aroma of well riped meat and together with the flavour from added spices, this type of product was tastier than common dried meat. The specific raw material composition and production conditions (natural and diverse animal feeding, smoking in traditional smokehouses, air drying) were some of the factors that contributed to the overall taste and specific sensory properties compared to industrially produced fermented sausages. National market today offers dry fermented sausages with similar sensory properties, but, unfortunatelly, overemphasized acidic flavour, often unacceptable for the consumers (Radetić, 1997; Petrohilou i Rantsios, 2005).
In order to preserve the quality of traditionally fermented sausages and to provide consistency in production process, we set the task to investigate sensory properties of „Užička“ sausage manufactured in traditional manner and to determine colour and firmness of the sausages using instrumental analysis.

MATERIALS AND METHODS

„Užička“ sausage was manufactured in traditional way in craft plant Brković Nikola on Zlatibor.
„Užička“ sausage was produced from beef of category I (50%) and II (20%), II category (20%) pork meat, firm fatty tissue (30%), nitrite pickle salt (2,50%), sacharose (0,33%) and spices (1,25%) (sweet and hot grinded paprika, black pepper and garlic). Frozen fatty tissue pieces were added to the cutter, followed by chilled meat, previously grinded up to 20mm granulation, while nitrite salt, sacharose and spices were added last. After the homogenisation and granulation of ∅ 8mm, mixture was stuffed into bovine small intestine ∅ 37-40 mm. Mixture temperature was -3°C. Sausages were hand shaped in the form of a horseshoe.
After the stuffing, sausages were left to drain and were kept in low humidity air in order for their surface to dry thus preparing for the smoking process. Sausages were smoked in traditional smokehouses using beech wood. Smoking, fermentation, ripening and drying lasted 21 days. „Užička“ sausage was produced in three intervals: November (the first fermentation - IF), December (the second fermentation – IIF) and January (the third fermentation – IIIF).
Using quantitative - descriptive test (Baltić, 1992; SRPS 6658, 2002), with grading scale from one to ten (1-unacceptable, 10-optimum), at the end of each fermentation, sensory properties of sausages were asessed (colour, surface cut, consistency – connection degree of meat and fatty tissue, fatty tissue quality, tenderness, and overall impression). Five persons panel was assembled in order to investigate sensory properties. Pannelists were previously tested for detection and recognition of various tastes (SRPS 3972, 2002) and odours (SRPS 5496, 2002).
Colour was determined according to CIE *L*, a*, b** system (*L** = light intensity, *a** = share of red, *b** = share of yellow) using Chromameter CR-400 (Minolta Co. Ltd.). Colour of cut surface was measured on three fresh cuts, three repetative measures taken at each surface.
Tenderness and firmness were determined on Instron 4301 instrument, by measuring cutting force and penetration force. Cutting force was determined using Warner-Bratzler contact acessory (force 0.25 kN, speed 100 mm/min). The samples were prepared by cutting 8 one-inch cylinders from each sausage using the mold. Penetration force was measured by five-pole needle (force 0.25 kN, speed 100 mm/min, sample thickness 10 mm). Samples were prepared by cutting the sausage with circular meat knife into 10 mm thick cylinders.
The results were statistically processed by calculating mean ($\overline{X}$) and standard devistion (Sd) for each sensory property. Coefficient of variation (Cv) was also calculated as relative

measure of dispersion showing the differences between multiple measurements. Statistical calculations were carried out using Microsoft Excell.

RESULTS AND DISCUSSION

Table 1 shows the results of sensory evaluation of "Užička“ sausage
"Užička“ sausage from first fermentation (IF) was manufactured in November, when the micro-climatic conditions of the smoking and drying were favorable, so the sausages from the first fermentation were evaluated with high grades for the properties studied and ranged from 8.3 to 9.00. Also, sausage from third fermentation (IIIF), produced in January, evaluated high grades for the properties studied which ranged from 8.00 to 9.00.
„Užička“ sausage from the second fermentation (IIF), produced in late December, were rated slightly lower scores, which ranged from 7.00 to 7.70.
It can be concluded that the climate conditions were favourable for sensory properties of fermented sausages. The most favourable conditions for manufacturing traditionally fermented sausages are in the period of the year with low temperatures and high relative humidity (Radetić, 1997).
The grades for color were the highest (8.50) in sausages produced in January (IIIF). Overall impression was also the highest in this group (8.90) comparing to sausages from IIF (8.80) and IF (7.50). Significant differences were observed in evaluation.
The results of sensory evaluation of “Užička“ sausage from IIIF were in accordance with the results obtained by the group of authors for similar product (Saičić et al., 2006; Vesković-Moračanin and Obradović, 2009). Morretti et al. (2004) evaluated sensory properties of traditional sausage from Sicily produced from meat of autochtonous pigs (Nero Siciliano), where ripening of one group was carried out in traditional way (Sicilian taverna), while the ripening of the second group was conducted in industrial conditions.
The authors concluded that the ripening of sausages in controlled conditions can be done during the entire year, while in traditional production, ripening cannot take place in summer time due to high temperatures.

Table 1. Results of sensory evaluation of „uzicka“ sausage at the end of production process

	I Fermentation			II Fermentation			III Fermentation		
Sensory properties	$\bar{X}$	Sd	Cv %	$\bar{X}$	Sd	Cv %	$\bar{X}$	Sd	Cv %
Colour	8,40	0,50	5,95	7,60	0,50	6,58	8,50	0,50	5,88
Surface of cut	8,30	0,45	5,39	7,40	0,42	5,65	8,70	0,27	3,15
Coherence	8,80	0,45	5,08	7,30	0,45	6,13	8,60	0,50	5,81
Fat tissue quality	8,40	0,42	4,98	7,30	0,45	6,13	8,00	0,35	4,42
Tenderness	8,90	0,42	4,70	7,10	0,22	3,15	8,20	0,50	6,10
Overall impression	8,80	0,27	3,11	7,50	0,50	6,67	8,90	0,42	4,70

$\bar{X}$ – mean
Sd – standard deviation
Cv– coefficient of variation

Table 2 shows the results of colour instrumental determination of "Užička“.
The results of cut surface colour show that sausage produced in November (IIF) were slightly lighter (L*-value) compared to sausages produced in December (IF) and January (IIIF),

however the differences were not statistically significant. The highest share of red was recorded in sausages from IIIF. Share of yellow (b*) had lower scores in sausages from IIF.

Table 2. The results of instrumental determination of cut surface colour of „Uzicka“ sausage at the end of production process in CIE L*a*b* sistem

	Light intensity *L**	Sd	Cv	Share of red *a**	Sd	Cv	Share of yellow *b**	Sd	Cv
I Fermentacion	45,36	3,57	7,87	15,52	1,64	10,6	7,34	0,81	11,04
II Fermentacion	43,82	4,16	9,49	17,83	1,58	8,86	7,22	0,62	8,59
III Fermentacion	35,26	2,37	6,72	19,27	0,93	4,83	7,88	1,11	14,09

$\overline{X}$ – mean
Sd – standard deviation
Cv– coefficient of variation

Table 3 shows the results of determination of firmness and tenderness of „Užička“ sausage from all three fermentations by measuring penetration force and cutting force using INSTRON 4301 instrument.

Table 3. Parameters of firmness and tenderness of „uzicka“ sausage at the end of production process

	Penetration force, (N)	Sd	Cv	Cutting force, (N)	Sd	Cv
I Fermentacion	23,55	3,57	15,2	44,37	5,62	12,7
II Fermentacion	18,88	1,96	10,4	39,64	1,78	4,49
III Fermentacion	12,49	1,67	13,4	24,84	2,91	11,7

$\overline{X}$ – mean
Sd – standard deviation
Cv– coefficient of variation

The highest penetration force (measure of firmness) was recorded in samples of „Užička“ sausage produced in November (IF), compared to sausages produced in December (IIF) and January (IIIF). However, higher cutting force was measured in sausages from IF and IIF compared to sausages from IIIF. The reasons for such results are to be found in different climate conditions. At the same time, higher meat content in fermented sausages results in higher firmness. This is corroborated by other authors (Vuković et al, 2009).
The impression is that the assessors overall process of ripening and drying of the sausage, in a third fermentation, should last for another ten days, so that the properties of the products tested in this fermentation, probably, got to a point higher ratings.

CONCLUSIONS

"Uzicka" sausages produced in November (first fermentation-IF) and produced in January (third fermentation-IIIF) had a very desirable sensory attributes, while the sausages produced in December (the second fermentation-IIF) were evaluated with slightly lower grades. Overall impression of "Uzicka" sausages from third fermentation (8.90) and first fermentation (8.80), had better assessment of overall impression than from second fermentation (7.50).
The results of instrumental determination of colour and firmness of „Užička“ sausage samples were in agreement with the results of sensory evaluation.
The investigated properties were better in sausages produced in January comparing to the ones produced in December and November.

On the basis of the obtained results it can be concluded that the best sensory properties of fermented sausages can be achieved when production is carried out in late winter which is the common period for traditional production of „Užička“ sausage.

ACKGNOWLEDGEMENT

The results are part of Integrated and Interdisciplinary Research Project No. III 46009, “Improvement and development of hygienic and technological procedures in producing food animal origin in order to obtain high-quality and safe products competitive on world markets” funded by the Ministry of Education and Science Republic of Serbia

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HYDROLYSIS OF SARCOPLASMIC PROTEINS DURING THE RIPENING OF TRADITIONAL *PETROVSKÁ KLOBÁSA* SAUSAGE

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ABSTRACT: In order to contribute to the knowledge about the proteolysis in *Petrovská klobása* the composition of water-soluble (pH 7.4) protein extracts of this traditional dry-fermented sausage was regularly examined as ripening progressed (120 days). Instead of widely utilized, classical SDS-PAGE, the Lab-on-a-Chip (LoaC), fast and reliable electrophoresis based method, was used to follow up the hydrolysis of sarcoplasmic proteins during the ripening. Water activity (aw), pH evolution and an increase of non-protein nitrogen (NPN) fraction were also followed up. Obtained results showed that *Petrovská klobása,* traditionally produced under low ambient temperature, undergoes slow processes of natural fermentation, drying and ripening. The pH decreased slowly from 5.67 (day 0) to 5.30 (day 60) ($P < 0.05$), what had an adverse effect on drying and proteolysis intensity. The aw gradually decreased from initial value of 0.95 to 0.86 ($P < 0.05$), while the NPN fraction increased up to day 90 (1.05 g/100g dm) and then stabilized further on. Throughout ripening slow degradation of sarcoplasmic proteins took place, resulting in disappearance of a few protein bands (≈16, 44 and 93 kDa) during second month of ripening.
Key words: *Petrovská klobása, sarcoplasmic proteins, hydrolysis, Lab-on-a-Chip electrophoresis*

INTRODUCTION

Traditional food products, which are linked with a place or region of origin, are closely connected to the culture and the identity of the local population. They carry a strong symbolic value important for the preservation of cultural identity, and are often perceived as higher quality. Consequently, an increased interest in traditional foods in Europe has been noticed in resent years (Pieniak et al., 2009), and the list of food products registered as PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) is continuously growing (http://ec.europa.eu/agriculture/quality/door/list.html). This applies also to dry-fermented sausages, which physical and sensorial characteristics are highly dependent on distinct cultural and social backgrounds of the populations, as well as on the environmental/climatic conditions in region of production. A large variety of these products (predominantly from France, Spain, Italy, Portugal, Greece and Slovakia), present on the European market, have been thoroughly characterized (Talon et al., 2008; Roseiro et al., 2008, 2010; Lücke and Vogeley, 2012).
On the other hand, the existing scientific information about Serbian dry-fermented sausages is still scarce, being important to improve the knowledge concerning the physicochemical and biochemical changes during production period. One of these sausages is *Petrovská klobasá*, traditional dry-fermented meat product which is a part of Slovaks' heritage, who inhabited northern part of Serbia (Vojvodina) in the second half of 18th century. Nowadays, *Petrovská klobasá* is produced in traditional manner, during winter (low ambient temperature) and without the use of additives and microbial starters. Drying and ripening process lasts about 4 months. Due to its specific flavor, aromatic and spicy-hot taste, dark red color and hard consistency *Petrovská klobása* is highly appreciated by consumers and it has been registered as PDO (Protected Designation of Origin) under Serbian law (Ikonić et al., 2010, 2012; Petrović et al., 2007; Tasić et al., 2012).

The proteolysis of sarcoplasmic and myofibrillar proteins is very important biochemical phenomena occurring during the ripening of fermented meat products. This phenomena is determined by enzymes originated both from muscle and microbial proteolytic system. It results in yield of several compounds, including polypeptides, peptides, amino acids, aldehydes, organic acids and amines, which participate in both flavour/taste and texture development (Dalmış and Soyer, 2008; Hughes et al., 2002; Roseiro et al., 2008; Spaziani et al., 2009). The changes undergone by the sarcoplasmic and myofibrillar proteins in fermented sausages during ripening have been widely studied using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). However, this electrophoretic method is time-consuming and consists of a number of necessary manual steps. On the contrary, microfluidic or Lab-on-a-Chip (LoaC) technology is a miniaturized electrophoresis based technique for fast, reliable and automated analysis of proteins on a chip. (Goetz et al., 2004; Soriano et al., 2003; Torbica et al, 2010).
In order to improve the knowledge concerning the proteolysis in *Petrovská klobása*, the present study reports the results for hydroliysis of sarcoplasmic proteins, obtained using LoaC electrophoresis. The samples were analysed at different times during drying and ripening up to 120 days, after the stuffing operation. Water activity (aw), pH and non-protein nitrogen (NPN) content were also followed up.

MATERIAL AND METHODS

Sausage preparation

Petrovská klobása dry fermented sausages were manufactured in traditional manner using hot-boned lean pork (80%) and pig fat (20%). After mincing the meat and the fat to a size of about 10 mm, raw materials were mixed with seasonings (red hot paprika powder - 2.5%, salt - 1.8%, raw garlic paste - 0.2%, caraway - 0.2% and sucrose - 0.1%). The seasoned batter was immediately stuffed in collagen casings (55 mm in diameter) and raw sausages were entirely processed in traditional smoking/drying room during 120 days.
Samples included seasoned batter prior to stuffing (0) and three randomly selected sausages at each sampling period (days 2, 4, 6, 9, 15, 30, 60, 90 and 120). Determinations of aw, pH and NPN were carried out at these sampling times, and the rest of the sausages were stored at -20 °C pending further analysis. Analyses for all samples were carried out in duplicate.

Water activity, pH and NPN

Water activity (aw) of samples was determined using Testo 650 measuring instrument with a pressure-tight precision humidity probe (Testo AG, USA). The pH was measured using the portable pH meter (Consort T651, Turnhout, Belgium) equipped with an insertion glass combination electrode (Mettler Toledo Greifensee, Switzerland). Dry matter (dm) was determined by drying the samples at 103 ± 2 °C to constant weight (ISO 1442:1997).
In order to determine non-protein nitrogen (NPN) 10 g of sausage were homogenized with 20 mL of 10% (w/v) trichloroacetic acid (TCA) for 1 minute at 13,500 rpm using an T18 Basic Ultra Turrax (IKA-Werke GmbH & Co. KG). The homogenate was left at +4 °C for 2 h, filtered and NPN was determined from 10 mL of filtrate by the Kjeldahl method (ISO 937:1978). The NPN fraction content was expressed as g/100 g dm of sample.

Extraction and LoaC electrophoresis of sarcoplasmic proteins

Sarcoplasmic protein extracts were prepared according to the method of Toldrá et al. (1993). Four grams of sausage were homogenized with 40 mL of 0.03 M potassium phosphate buffer (pH 7.4) for 2 min using an Ultra Turrax at 13,500 rpm. The homogenate was centrifuged for 20 min at 10,000g at 4 °C. The supernatant contained the sarcoplasmic proteins. The protein concentrations of sarcoplasmic protein extracts were determined by method of Lowry et al. (1951), using bovine serum albumin as standard protein, and adjusted with deionised water to give a final concentration of 4 mg/mL.
The chip-based separations were carried out as described by Torbica et al. (2010), using Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) in combination with the

Protein 230 Plus LabChip Kit and the dedicated 2100 expert software. The chips were prepared according to Agilent Protein 230 Kit Guide (assay protocol). The complete analysis of 10 protein samples, including sizing and quantification, takes 25 minutes (including the start-up phase of the instrument).

Statistical analysis

One way (ANOVA), Post-hoc (Duncan test) was performed using the software package Statistica 9.1 for Windows, Stat Soft, Tulsa, Oklahoma, USA. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The results of aw, pH and NPN fraction content are presented in Table 1. During the processing of *Petrovská klobása* considerable drying occurred. The aw gradually decreased from initial value of 0.95 to 0.86 ($P < 0.05$). The drying pattern was determined by the influence ambient conditions in traditional smoking/drying room (low air temperature and high relative humidity) which caused slow dehydration of sausage. Likewise, the low environmental temperature adversely affected the growth and activity of the indigenous lactic acid bacteria (LAB) and consequently the intensity of fermentation, as it was previously found in French traditional dry sausages (Rason et al. 2007). Thus, Danilović et al. (2011) reported the highest number of LAB, about 10^7 CFU/g, only after 15 days of ripening in *Petrovská klobása,* made in the same time and from the same raw meat/fat mixture. Obviously, the low growth rate of LAB is consistent with pH profile (Table 1). The fermentation period was prolonged and the pH reached its lowest value (5.30) after 60 days of production, when it started gradual increase due to degradation of proteins, as it was proposed in previously published work of Spaziani et al. (2009). Furthermore, relatively high pH additionally influenced lower intensity of drying since the isoelectric point (pI) of myofibrillar proteins is reached after two months of ripening. At pI the water holding capacity of myofibrillar proteins is the lowest and it can be easily exudated from the muscle fibers and evaporated (Huff-Lonergan, 2002).

Table 1. Changes in aw, pH and NPN fraction content (expressed as g/100 g dm) during the ripening of *Petrovská klobása.*

Ripening time (day)	0	2	4	6	9	15	30	60	90	120
aw	0.952^{b}	0.949^{ab}	0.946^{ab}	0.940^{abc}	0.939^{abc}	0.935^{ac}	0.927^{c}	0.902^{f}	0.887^{e}	0.860^{d}
pH	5.67^{g}	5.63^{f}	5.60^{e}	5.56^{ae}	5.53^{a}	5.48^{cd}	5.47^{c}	5.30^{b}	5.33^{b}	5.52^{ad}
NPN	0.66^{b}	0.69^{ab}	0.70^{a}	0.70^{a}	0.79^{d}	0.82^{e}	0.85^{f}	0.90^{g}	1.05^{c}	1.04^{c}

$^{a-g}$ Means within the same row with different superscript letters are different ($P < 0.05$).
NPN – non-protein nitrogen

The NPN increased progressively up to 90 days of ripening and then stabilised further on. Several authors have reported that proteolysis during the ripening of fermented sausages is followed by an increase in NPN concentration (Candogan et al., 2009; Dalmış and Soyer, 2008; Toldrá, 2002).

Proteolysis of sarcoplasmic proteins

The electrophoretic profiles of sarcoplasmic proteins extracted from *Petrovská klobása* during the ripening are shown in Fig. 1.

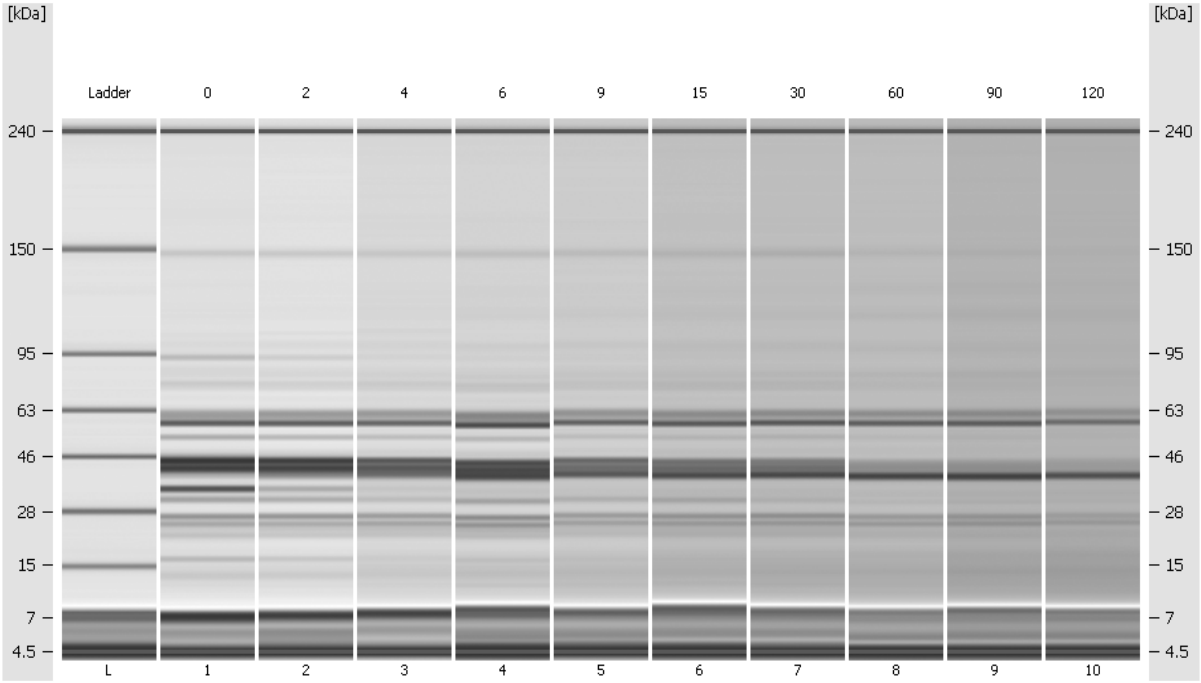

Figure 1. Gel image of electrophoretically separated sarcoplasmic proteins during the ripening of *Petrovská klobása* (lanes 1-10). Lane L (Ladder), molecular weight standards ranging from 4.5 to 240 kDa

The sarcoplasmic proteins, ranging in molecular weight from ≈14 kDa to ≈148 kDa, were already extracted from the seasoned butter (before stuffing) (Table 2). During the ripening period polypeptides with molecular weights of ≈14, 23, 25, 58 and 62 showed a relevant increase, while six new protein bands were detected after 2 (≈40, 99 kDa), 6 (≈115 kDa) and 15 (≈15, 18, 129 kDa) days. Furthermore, the intensity of ≈40 kDa band progressively increased throughout ripening, and after 120 days it was the most common polypeptide in sarcoplasmic proteins extract. This result is in accordance with finding of Martín-Sánchez et al. (2011). Formation and increase of some low molecular weight bands (<20 kDa) has been reported by a number of authors (Dalmış & Soyer, 2008; Hughes et al., 2002; Martín-Sánchez et al., 2011; Spaziani et al., 2009).

Throughout ripening period slow degradation of sarcoplasmic proteins took place. Hence, polypeptides with molecular weights of ≈32, 42 and 53 lost about 50% of their initial concentration only after one month of processing, while protein bands at ≈16, 44 and 93 kDa completely disappeared in next 30 days. On the contrary, the intensity of the protein band with molecular weight ≈35 kDa decreased markedly after only 2 days. Others (Casaburi et al., 2007; Dalmış and Soyer, 2008; Martín-Sánchez et al., 2011; Spaziani et al., 2009) evaluating the proteolysis in fermented sausages found similar low-intensity degradation of sarcoplasmic proteins in products without starter culture as a result of weak activity of endogenous proteases.

In the case of *Petrovská klobása,* the activity of these enzymes was probably negatively affected by relatively high pH (Table 1) and low processing temperature as it was previously reported by other authors (Casaburi et al., 2007; Roseiro et al., 2008). Moreover, dominant lactic acid bacteria strains isolated from this sausage (*Lactobacillus sakei, Leuconostoc mesenteroides and Pediococcus pentosaceus*) showed variable or no proteolytic activity during the ripening (Danilović et al., 2011), confirming a minor role of bacterial proteinases in proteolysis process (Toldrá, 2002).

Table 2. Share (%) of electrophoretic bands in total sample of sarcoplasmic proteins extracted from *Petrovská klobása* during the ripening

MW (kDa)	Ripening time (day)									
	0	**2**	**4**	**6**	**9**	**15**	**30**	**60**	**90**	**120**
13.9	2.4	2.8	4.0	4.0	5.5	4.3	5.2	5.4	5.6	3.7
14.6	-	-	-	-	-	1.9	2.6	2.8	3.3	9.0
16.6	2.4	1.8	1.4	2.0	2.5	2.1	2.1	-	-	-
17.5	-	-	-	-	-	1.2	1.9	3.9	5.6	9.0
22.6	1.9	1.6	2.1	2.0	2.5	2.9	2.8	3.8	3.0	4.5
25.1	4.1	5.0	6.1	5.7	5.6	5.5	5.7	5.7	6.1	5.5
26.9	5.1	5.6	5.8	5.8	5.5	5.8	6.0	5.0	5.2	4.0
31.8	3.8	3.5	2.7	4.0	3.4	3.4	1.6	0.8	0.9	0.7
34.5	13.0	3.9	2.1	1.5	1.4	0.9	1.2	1.1	1.1	1.1
40.0	-	6.8	14.2	19.0	22.2	24.2	24.1	30.7	32.5	29.5
42.1	23.0	23.1	20.3	13.9	11.7	10.7	11.4	10.9	8.3	7.5
44.3	23.3	22.1	16.0	14.9	13.3	9.7	7.7	-	-	-
53.1	2.2	1.9	1.5	1.3	1.0	0.9	1.0	0.6	0.5	0.3
58.3	9.7	11.0	12.1	13.2	12.5	12.9	13.3	14.2	14.4	13.2
61.6	4.9	6.5	7.6	7.8	7.6	7.5	7.0	8.0	7.6	7.1
78.1	1.1	1.1	1.4	1.5	1.5	1.6	1.5	1.3	1.0	0.5
83.2	0.6	0.7	0.7	0.7	0.8	0.9	0.9	1.0	1.2	1.0
93.1	1.6	0.9	0.3	0.2	0.1	0.2	0.1	-	-	-
98.8	-	0.4	0.4	0.5	0.6	0.7	0.8	1.1	0.8	0.6
115.2	-	-	-	0.4	0.6	0.7	0.7	1.3	1.3	1.3
129.4	-	-	-	-	-	0.5	0.5	1.1	0.8	1.0
147.9	0.9	1.3	1.3	1.6	1.7	1.5	1.9	1.3	0.8	0.5

CONCLUSIONS

The findings of this study show that *Petrovská klobása,* traditionally produced under low ambient temperature, undergoes slow processes of natural fermentation, drying and ripening. The pH drop was slight, adversely affecting drying and proteolysis intensity. Consequently, slow degradation of sarcoplasmic proteins took place, resulting in notable decrease and disappearance of a few protein bands after 30 days of ripening.

ACKNOWLEDGEMENTS

The authors wish to express their sincere gratitude to the Ministry of Education and Science of the Republic of Serbia for its financial support (Project: TR 31032).

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BIOGENIC AMINES CONTENT AS AN INDICATOR FOR THE ESTIMATION OF GOOD MANUFACTURING PRACTICE DURING *PETROVSKÁ KLOBÁSA* PRODUCTION

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ABSTRACT: In this paper, good manufacturing practice (GMP) during *Petrovská klobása* production, throughout three seasons and for the total of 13 batches (A1, A2, B1, B2, B3, B4 – first; C1, C2, C3 – second; D1, D2, E1, E2 – third season), was estimated based on tyramine, histamine, phenylethylamine, vasoactive and total biogenic amines content. Analyses were performed by HPLC-DAD on Eclipse XDB-C18 column. Chromatographic separation of dansyl chloride derivatizated amines was completed in 8 min.
Histamine, as the most important amine from the food safety point of view, was not detected in any analyzed sample. Total content of biogenic amines ranged from 77.8 to 174 mg/kg in the first, from 456 to 744 mg/kg in the second and from 144 to 217 mg/kg in the third production season. Tyramine was not detected in A1 batch, while in others its content ranged from 6.90 (B1) to 160 mg/kg (C3).
For C batches content of phenylethylamin, as well as the sum of vasoactive biogenic amines was found to be higher than values considered as upper limits (30 mg/kg; 200 mg/kg, respectively) for indication of GMP. According to obtained results good manufacturing practice was applied during production of *Petrovská klobása* in the first and third season, while in the second season that was not the case.
Key words: *Biogenic amines, Traditional fermented sausage (Petrovská klobása), Good manufacturing practice*

INTRODUCTION

Petrovská klobása is traditional fermented sausage which has been produced for over 250 years in the area nearby town of Bački Petrovac in the Autonomous Province of Vojvodina, Republic of Serbia. It is produced exclusively from pork meat and fat, red hot paprika powder, salt, crushed garlic, caraway and sugar and is characterized by specific hot taste, aromatic and spicy flavour, dark red color and hard consistency (Petrović et al., 2007; Ikonić et al., 2010; Tasić et al., 2010).
Traditional fermented sausages are characterized by handmade manufacturing usually in small-scale units, following spontaneous fermentation by their particular in-house flora (Talon et al., 2007). In contrary to industrial production, traditional products are manufactured with traditional technologies generally without adding starter cultures and the process of drying and ripening is carried out in rooms with less temperature, relative humidity and air velocity control (Roseiro et al., 2010; Lebert et al., 2007b; Parente et al., 2001). This process favours the growth of autochthonous microflora, originated from the raw materials or environment, which influences the flavour, texture, nutritional qualities, safety, and other characteristics of this type of sausage. (Moretti et al., 2004; Latorre-Moratalla et al, 2008; Lebert et al., 2007a). Also, type of microflora that develops during fermentation, drying and ripening is closely related to the diversity in formulation as well as technological conditions applied during these processes (Latorre-Moratalla et al, 2008; Suzzi & Gardini, 2003). Small producers of traditional products may encounter technical and financial difficulties to comply with official food safety regulations established for industrial processes, such as the hazard analysis and critical control point (HACCP) plan (Latorre-Moratalla et al, 2008, Lu et al., 2010).

The ripening of fermented sausages offers favourable conditions for biogenic amines production due to activity of present and developed microflora, acidification and the proteolysis that increases the amounts of free amino acids available for decarboxylation (Halász et al., 1994; Santos et al., 1996; Shalaby, 1996; Suzzi & Gardini, 2003; Latorre-Moratalla et al, 2008; Latorre-Moratalla et al, 2010).
Biogenic amines are associated with several health disturbances: hypertensive crises, headache, nausea, vasoactivity, and allergic reactions due to its toxicity (Hernández-Jover et al., 1996; Vidal-Carou et al., 1990). Also, they are of concern in relation to food spoilage or ripening stage (Önal, 2007; Roserio et al., 2006; Bover-Cid et al., 2001; Hernández-Jover et al., 1997). The level of biogenic amines in meat and meat products has been used as a quality index of unwanted microbial activity (Lu et al., 2010), as well as for a good manufacturing practice evaluation (Latorre-Moratalla et al, 2008; Eerola et al., 1998; Santos et al., 1996; Shalaby, 1996). Thus, interest in biogenic amines content lies in safety and quality issues (Latorre-Moratalla et al, 2008).
Total biogenic amines content in food considered as dangerous is 1000 mg/kg according to Santos et al., (1996). Shalaby (1996) suggested levels of few amines as parameters for the evaluation of good manufacturing practices (GMP): tyramine 100-800 mg/kg, histamine 50-100 mg/kg, phenylethylamine <30.0 mg/kg, while Eerola et al. (1998) proposed that sum of vasoactive biogenic amines (tyramine, histamine, tryptamine, phenylethyamine) as a possible indicator of hygienic conditions and GMP in the sausage production, which should not exceed 200 mg/kg. Development of appropriate manufacturing technologies to obtain sausages free or nearly free from biogenic amines is one of the current targets of the meat sector, including the traditional manufacturers (Latorre-Moratalla et al., 2010).
The aim of this study was to determine the content of total biogenic amines, content of tyramin, histamine, phenylethylamine and sum of vasoactive biogenic amines as possible indicators of good manufacturing practice (GMP).

MATERIAL AND METHODS

Sausage preparation

All sausage mixtures were made of lean pork meat and fat 80:20, 2.50% red hot paprika powder, 1.80% salt, 0.20% crushed garlic, 0.20% caraway and 0.15% sugar in traditional manner. Pork meat and fat were grounded to a 10 mm particle size and all ingredients were mixed approximately 10 min using traditional technique. Afterward, the mixtures were stuffed in natural or collagen casings and raw sausages were entirely processed in traditional smoking/drying room or, alternatively, in an industrial ripening room (B3 and B4) (Table 1).

Table1. Raw material, casings, processing conditions and the end of drying for examined batches

Batches	Raw material	Casings	Processing conditions		End of drying (day)
			Smoking	Drying	
A1	hot deboned meat (2h *post mortem*)	natural	traditional	traditional room	90.
A2		collagen			
B1	cold meat (24h *post mortem*)	natural			
B2		collagen			
B3		natural	industrial	industrial ripening chamber	45.
B4		collagen			
C1	cold meat (24h *post mortem*)	natural	industrial	industrial ripening chamber	60.
C2		collagen			
C3		collagen			
D1	cold meat (24h *post mortem*	collagen	traditional	traditional room	90.
D2					
E1			industrial	industrial ripening chamber	60.
E2					

Traditionally processed sausages were smoked using cool procedure for 10 days with pauses, while sausages processed in industrial conditions were smoked for 6 h (12 30 min), during 3 days.
According to Serbian legislation (Serbian Regulations, 2004) moisture content for dry fermented sausages has to be less than 35.0%. Sausages dried in traditional room needed 90 days to reach required moisture content, while sausages dried in industrial ripening chamber reached this value after 45 or 60 days, what determined the sampling of the sausages.
At the end of drying period samples were homogenized and stored at -20 °C until analyzed.

Biogenic amines analysis
Tryptamine, phenylethylamine, putrescine, cadaverine, histamine, serotonin, tyramin, spermidine and spermine were determined as their dansyl derivatives following the high-performance liquid chromatography. Sample preparation and extraction were done according to Eerola et al., 1993. HPLC analysis was performed by using a liquid chromatography (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies), a binary pump, an online vacuum degasser, an auto sampler and a thermostated column compartment, on an Agilent, Eclipse XDB-C18, 1.8 µm, 4.6 x 50 mm column. Solvent gradient was performed by varying the proportion of solvent A (acetonitrile) to solvent B (water) as follows: initial 50% B; linear gradient to 10% B in 7.6 min, 10% B to 10 minutes; linear gradient to 50% B in 2 min. System was equilibrated 3 minutes before next analysis. Flow rate was 1.5 mL/min., column temperature was 40 °C and 5 µL of sample was injected. The spectra were acquired in the range 190-400 nm (Tasić et al., 2012).
Detection limits of the amines were determined to be 0.1 µg/g for putrescine and spermidine, 0167 µg/g for cadaverine and tyramin, 0.25 µg/g for tryptamine, phenylethylamine and histamine and 0.5 µg/g for serotonin and spermine.

Statistical analysis
One way (ANOVA), Post-hoc (Duncan test) was performed using the software package Statistica 9.1 for Windows, Stat Soft, Tulsa, Oklahoma, USA. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The contents of biogenic amines in traditional dry fermented sausage *Petrovská klobása* during three production seasons, referred to fresh matter, are presented in Table 2.
Limit of 1000 mg/kg for total biogenic amines content considered by Santos (1996) was not exceeded at the end of drying period in any analyzed sample. The content of total biogenic amines was significantly higher ($P < 0.05$) in the sausages produced in the second season, averagely ranging from 542 to 684 mg/kg, comparing with first and third seasons, but still lower than proposed limit. Shalaby (1996) suggested levels of few amines as parameters for the evaluation of good manufacturing practice: tyramine 100-800 mg/kg, histamine 50.0 - 100 mg/kg, phenylethylamine < 30 mg/kg. Average contents of tyramine did not exceed the recommended values in all analyzed groups, and were in a range from ND to 17.3 mg/kg; from 100 to 160 mg/kg and from 25.4 to 80.3 mg/kg for first, second and third season respectively. Histamine was not detected in any of the investigated sausage groups. Unlike tyramine and histamine, some groups of sausages had greater average content of phenylethylamine than recommended values. So, in groups A1 and A2, made in the first season from hot deboned meat (cca 3 h *post mortem*), the average content of this amine were 51.6 mg/kg and 33.2 mg/kg, respectively. This finding may be one of the reasons why *Petrovská klobása* should be, in the future, made exclusively from cold meat (24 h post mortem). Furthermore, in all investigated groups of sausages made in the second season phenylethylamine content was higher than the proposed 30 mg/kg (C1 30.4 mg/kg; C2 60.2 mg/kg, C3 42.4 mg/kg). Phenylethylamine was not detected in sausages made in third production season. The limit of 200 mg/kg for the sum of vasoactive biogenic amines was

proposed by Eorola et al., (1998) as an indicative criterion for good hygienic conditions and good manufacturing practice. This limit was not exceeded in any sample at the end of drying in the first and third season. On the other hand, average content of the sum of vasoactive biogenic amines in the second season exceeded this limit and were in the range 248-458 mg/kg.

Table 2. Biogenic amines content in traditional dry fermented sausage *Petrovská klobása* as possible indicators of good manufacturing practice

Batch	**Santos (1996)**	**Shalaby (1996)**			**Eorola et al. (1998)**
	total	tyramine	histamine	phenylethylamine	sum of vasoactive
	< 1000 (mg/kg)	100-800 (mg/kg)	50-100 (mg/kg)	< 30.0 (mg/kg)	< 200 (mg/kg)
A1	113[b]	ND	ND	51.6	66.4
A2	164[d]	17.3	ND	33.2	103
B1	174[d,e]	6.90	ND	28.6	73.6
B2	163[d]	16.8	ND	29.4	121
B3	77.8[a]	7.34	ND	ND	36.1
B4	86.5[a]	14.8	ND	11.8	52.8
C1	585[h]	146	ND	30.4	452
C2	542[g]	100	ND	60.2	330
C3	684[i]	160	ND	42.4	248
D1	140[c]	29,7	ND	ND	70,0
D2	151[d]	25,4	ND	ND	63,7
E1	217[f]	80,3	ND	ND	126
E2	181[e]	38,5	ND	ND	90,2

In the same column, different letters means that values are significantly different ($P < 0.05$) Results are expressed as means
ND e not detected.

Based on the obtained results, it can be ascertain that in the first and in the third season there were no deviations from good manufacturing practice in *Petrovská klobása* production. Further on, in the second season there was deviation from good manufacturing practice, although in all investigated sausage groups total content of biogenic amines was below the recommended values (<1000 mg / kg).
During drying and ripening of *Petrovská klobása* differences in biogenic amines type and content were found between groups. These differences could be explained by development of different microflora as well as different time of drying and ripening and with complex interactions of many factors, among which raw materials (hot deboned meat or cold meat), type of casings, appropriate drying model and their interactions certainly take an important role.

CONCLUSIONS

The results obtained in this study indicated no evidence of deviations from good manufacturing practice in Petrovská *klobása* production in the first and in the third seasons, while in the second season such deviation was observed.

ACKNOWLEDGEMENTS

The authors wish to express their sincere gratitude to the Ministry of Education and Science of the Republic of Serbia for its financial support (Project: TR 31032).

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THE ANALYTICAL AND NUTRITIONAL STUDY OF THE ALBANIAN AUTOCHTHONOUS TABLE OLIVE *KOKËRMADH BERATI*

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ABSTRACT: Olive tree is one of the most important plants related to the economical interest in Albania. Eventually it is one of the study objects, which constitutes a cultivation area of more than 40 000 ha. Studies with focus the olive tree mainly have been of statistical profile comprising, (yield, area of plantation and their exploitation) and botanical (morphological determinations of olive cultivars). Recently has been growing interest on the analytical studies of chemical compositions.
One of the main olive autochthonous cultivars used as a Table olive is the *Kokermadh Berati* cultivated in the region with same name. It contributes by 20% of the total fund of the ~6 million olive trees. This study has analyzed samples from the crop year 2010-2011. It evaluated the oil content, fatty acid profiles, total phenol content and antioxidant activity. GC analysis of fatty acids revealed that the oleic acid content resulted in 76.26%. The content of palmitic acid is relatively low, 10.41%). The level of linoleic acid is considered relatively low (6.92%). The content of the stearic acid resulted 2.20%, while the linolenic acid resulted 0.67%. The Saturated Fatty Acids (SFA) group resulted in 12.92%, the Monounsaturated Unsaturated Fatty Acid (MUFA) content to 79.53% and the Poly Unsaturated Fatty Acid (PUFA) 7.59%. The nutritional value of n-6/n-3 show very interesting values by 10.31. The Total Phenol Content (TPC) expressed as Gallic Acid Equivalency (GAE) resulted 226.97 ±1.40 mg GAE/kg Olive Oil.
Key words: *Kokermadh Berati, Table Olive, Fatty Acid, Total Polyphenol Content*

INTRODUCTION

Cultivation of olive tree in Albania is mainly concentrated in the Western part of the country across the Adriatic and Ionian seas. A number of archeological evidences give indication of the olive cultivation since ancient times (Kafazi and Muço, 1984; Thomaj and Panajoti, 2003). Production capacity varies in 70 000 ton olives, 10000-11000 ton olive oil. The area of planted olive trees is approx. 41000 ha, or 6.3% of the arable land (MBUMK, 2009). This area is distributed in 90 000 farms (MBUMK, 2009). Incomes from this sector vary to 28 million Euros (FAOSTAT, 2011). According to FAOSTAT, Albania is ranked 20th in olive and olive commodities in the world (FAOSTAT, 2011). From the above mentioned data can deduct that the quantity of Table Olive is calculated to 14000 ton (IOOC, 2010).
Scientific studies have concluded that exist 22 autochthonous olive cultivars (Thomaj and Panajoti, 2005).The olive groves are situated mainly in hilly and mountainous lands. A continuous selection throughout the centuries has created that actual structure of olive cultivars. Olive cultivars in Albania are classified in two groups based on the plantation area and their importance: principal and secondary olive cultivars. Principal cultivars are dominant in considerable areas in many regions, while secondary cultivars are not dominant in their region but are localized in some groves. There are six main regions of olive groves that originate since antiquity: *Kalinjoti* (Vlora), *Kokërrmadh Berati* (Berati), *Mixan* (Elbasani), *Ulliri Bardhë Tirana* (Tirana), *Krypsi Krujës* (Kruja), *Kallmet* (Lezha) (Frezzoti, 1930; Kafazi, 1980; Ismaili and Celoaliaj, 1995; Thomai and Panajoti, 2004).
Kokërrmadh Berati (Berati) is a cultivar that is used as Table Olive, and is named according to the name of region. It is a self sterile cultivar, and mainly cultivated in Districts of Berati, Fieri and Lushnja. The fruit' weight varies 6-9 g. The stone weight varies to 0.7 g, and the

flash ray varies to 89.3 %. It is characterized from an average and periodic productivity. The oil content varies 18-21%. The oil quality is good. It is considered of moderate consistency to the cold weather. It is classified as the best autochthon table olive cultivar in Albania.

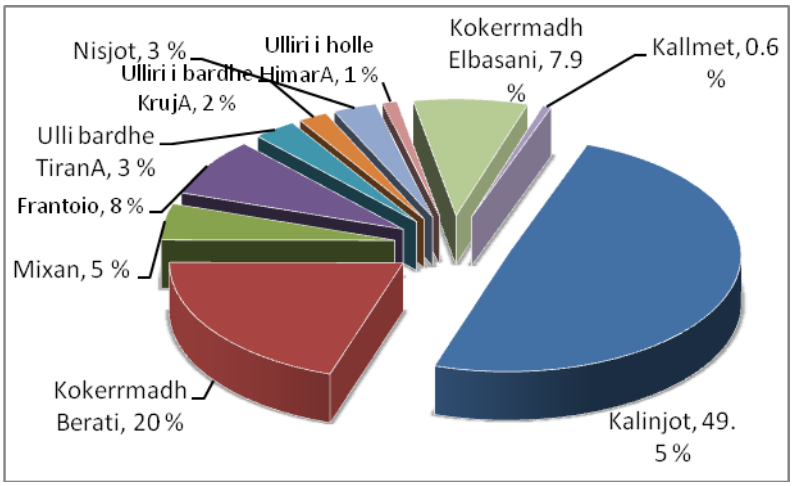

Figure 3: Structure of olive cultivars in Albania

The Berati region owns 6702 ha of olive plantations. The Kokermadh Berati contributes by 90% of overall trees.

Fatty acid profile characterization is used to give a differentiation between olive cultivars as well as to evaluate the nutritional aspects of this product (Boskou, 2006; Mannina *et al.,* 2003).

This study has analyzed the Fatty Acid Profile, Total Phenolic Content for the *Kokermadh Berati* olive cultivar. It is the first study on that cultivars and it has interest for scientific community to make present this cultivar, which is very important in national level, which counts for above 1.2 million planted trees (MBUMK, 2009).

MATERIAL AND METHODS

Olive samples and extraction

Samples of Kokermadh Berati were harvested at the same time (November 2010) from the Berati region. The harvested olive fruits were according to the appropriate maturity index suggested for olive oil extraction period. Oil extraction was carried out using extraction conditions similar to those used at industrial scale, using a SPREM Oliva Press (Italy) in the laboratory. The oil was separated by decanting, transferred into dark glass bottles and stored in the darkness at 4°C until further analysis.

Analytical methods

Fatty acid methyl esters (FAME) were prepared through direct acidic transesterification, as originally proposed originally by Lepage and Roy (1984) and later modified by Carvalho and Malcata (2005), using pentadecanoic acid as Internal Standard. The assay of FAME was carried out with a HP-6890 Gas chromatograph, equipped with a Flame Ionization Detector (GC-FID). Calculations were performed according to AOCS Official Method Ce 1b-89 (Fyrestone, 1994). Identification of fatty acids was undertaken with pure standards (Sigma-Aldrich, Supelco), based on the comparison of retention times. Fatty acids were calculated as the percentage of the total fatty acids. Each sample was analyzed in triplicate.

Total Polyphenolic content (TPC) was measured by a colorimetric method proposed by Kalantzakis et al. (2006). Briefly, samples were dissolved in n-hexane (Sigma, Germany) and extracted with a methanol/water mixture (60:40, vol vol^{-1}). The insoluble fraction (non-polar) in methanol/water fraction was removed, whereas the polar fraction was used, as it was, for further analysis. The absorbance of mixture was measured in UV-VIS Mini-1240

Spectrophotometer (Shimadzu) at 725 nm. Results were expressed as Gallic acid equivalent (mg kg^{-1} olive oil), calculated from the following calibration curve, determined by linear regression, where [GA] is the concentration of Gallic acid, expressed as mg kg^{-1} olive oil: A_{725}= 3.015 [GA] + 0.005 (r^2=0.999). Each sample was measured in triplicate.

Statistical analysis

The complete data was evaluated by randomized block design, with three replicates from fatty acid analysis and duplicates for TPC values. Results were displayed as mean values and standard error (n=3). Significance of the differences among the values was determined by Analysis of Variance using One-way ANOVA test, with a level of significance at $P<0.05$.

RESULTS AND DISCUSSION

Fatty acid composition

The major fatty acids present were: oleic (C18:1), palmitic (16:0), linoleic (C18:2) and stearic acid (C18:0), (Table 1). Results revealed that the fatty acid content falls in the average percentage intervals described by IOOC (2011). Palmitic acid resulted in 10.41%, Stearic acid 2.10 %. The content of Linoleic acid resulted in 76.26%.The content of linolenic acid (LA) varied from 6.92%.

Results showed that there was a similarity among Kokermadh Berati and other Albanian studied cultivars (Topi *et al*., 2011) and other cultivars from Italy and Greece (Aparicio and Luna, 2002; Motilva *et al.,* 2000; Bouaziz, Chamkha, Sayadi, 2004). Comparison of FA profile of Kokermadh Berati with FA profiles of cultivars from Southern coast of Mediterranean sea, showed the same trend where FA profile present high content of polyunsaturated fatty acids (Pinelli *et al*., 2003; Haddada *et al.*, 2008).

n-6/n-3 ratio

Olive oil is an important source of healthy lipids. The proposed ratio **n-6/n-3** = 4, respectively between unsaturated acid with two and three double bound, gives an indication on the quality of the lipid source regarding to nutrional aspects (DGA, 2010). The *Kokërrmadh Berati* presents a ratio **n-6/n-3** of 10.31.

Table 1: Fatty acid composition, SFA, MUFA, PUFA (in % FAME) and n-6/n-3 ratio

Formula	Average±Stdev
16:0	10.41±0.12
16:1(n-9)	0.13±0.04
16:1(n-7)	0.61±0.07
17:0	0.00±0.00
17:1 (n-7)	0.00±0.00
18:0	2.10±0.10
18:1(n-9)trans	0.00±0.00
18:1(n-9)cis	76.26±0.56
18:1(n-7)	2.20±0.11
18:2 (n-6)cis	6.92±0.15
20:0	0.40±0.04
18:3 (n-3)	0.67±0.02
20:1 (n-9)	0.13±0.03
22:0	0.20±0.08
n-6/n-3	10.31
SFA	12.92
MUFA	79.53
PUFA	7.59
18:1/18:2	11.02
MUFAs/SFAs	6.16
MUFAs/PUFAs	10.48

Analysis of Saturated and Unsaturated Fatty Acids groups

Analyzing the fatty acid profiles based on their family, as saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids can be used to distinguish cultivars among each others. Regarding to SFA, this cultivars show good profile, a low content of Saturated Fatty acids. The MUFA value resulted high (79.53%) while the PUFA content very low, 7.59%.

Polyphenol content

Total Polyphenol Content (TPC) for *Kokërrmadh Berati* cultivar resulted 125.6± 6.09 mg kg^{-1} GA (olive oil). Result show major differences compared to other native cultivars (Topi *et al.,* 2011), which may be correlated to the cultivar itself, rather than to agriculture practices or other factors. The comparison of TPC content with other Italian cultivars, reveal that this cultivar shows similarity with cultivars from the Toscana Region, Italy; and Dalmatian Coast, Croatia (Pinelli et al., 2003).

CONCLUSIONS

Analysis of virgin olive oil from native cultivars of Albania is rather important, not only regarding to the scientific interest in characterizing native plants from a specific region, as well as to identify the origin of the virgin olive oils in the market. Study of the chemical composition of the Table olive Kokermadh Berati is important not only to the scientific collection data, but also related to the nutritional aspect. The results of this study are a contribution to the characterization of the Albanian monovarietal olive oils, which should be completed with more samples obtained from different crop seasons.

ACKNOWLEDGEMENTS

The first author acknowledges CBQF - Escola Superior de Biotecnologia – Universidade Católica Portuguesa for having hosted the experimental work.

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THE DYNAMICS OF THE FERMENTATION PROCESS AND SENSORIAL EVALUATION OF SAUERKRAUT, CULTIVAR FUTOŠKI AND HYBRID BRAVO- COMPARATIVE STUDY

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ABSTRACT: Preservation of foods by fermentation is a widely practiced and ancient technology. Lactic acid bacteria because of their unique metabolic characteristics are involved in many fermentation processes of milk, meats, cereals and vegetables. The fermentation of white cabbage into sauerkraut traditionally proceeds in the presence of salt. White cabbage, cultivar Futoški has protected geographical origin according to domestic legislations because of its specific physical and sensory characteristics, as native as well as sauerkraut. The objective of this study was to follow the dynamics of biofermentation parameters for white cabbage, cultivar Futoški and hybrid Bravo. These two varieties were spontaneous fermented traditionally with addition of salt in concentrations of 2%. Fermentation process was conducted under anaerobic conditions at temperature of 18^{o}C. Organic acids and pH were determinate in defined time intervals during fermentation process. Cabbage heads were also sensorially evaluated in native form before the beginning of fermentation as well as sauerkraut. Sensory evaluation was performed by 4 trained panelists in four measurements. Experiment showed that white cabbage, cultivar Futoški has more acceptable sensory characteristics and faster achievement of completion of fermentation of cabbage tissue.
Key words: *cabbage, sauerkraut, fermentation, sensory, salt*

INTRODUCTION

Increasing consumer demands for healthful foods have fostered the development of an active functional foods market. Numerous vegetable foods are already associated with health promotion and disease prevention. One group of vegetables that has been widely regarded for their antioxidant and anticarcinogenic properties are Brassica vegetables, including all cabbage-like vegetables (Chyou et al.,1990; Higdon et al., 2007). One of the most important commercial products obtained from Brassica vegetables is sauerkraut, which results from the lactic acid fermentation of shredded and salted white cabbage. It has usually been prepared by spontaneous fermentation caused by the lactic acid bacteria (LAB) present on cabbage leaves (Leuconostoc mesenteroides and Lactobacillus plantarum, predominantly) in a correct succession (Holzapfel et al., 2003). This process represents a cheap cabbage preservation method, and also consumers appreciate traditionally fermented products for their outstanding gastronomic qualities (Penas et al., 2010). In Serbia and Vojvodina Futog is well known region where the quality cabbage is cultivated. White cabbage, as raw material has been used like grouser for salads and thermally treated meals during most of the year. During the winter cabbage carried out the biofermentation process and had been storaged like so cold „sauerkraut“ (Niketić-Aleksić G,1988). Traditional foods are an expression of culture, history and lifestyle and generally possess health qualities, since tradition rarely favors foods which are not palatable and healthy. White cabbage, cultivar Futoški has got certificate as product with geographic origin according domestic legislation (Mastilović et al., 2008). White cabbage, cultivar Futoški is traditional population in Futog district, it has some speciphic morphological characteristics like loose heads, specific oval head and thin elastic leaves as

well as slightly expressed nervature of leaves compared to hybrid Bravo F1. Salt addition is a critical factor during cabbage fermentation, because the microbial growth and sensory properties of the final product are affected by the amount of salt used. The salt content in sauerkraut usually ranges between 0.6 and 2% NaCl, but it can even exceed 2%. Pederson and Albury (1969) defined the optimum conditions for fermentation of cabbage into sauerkraut to be uniform salting of the shredded cabbage with 2.0% to 2.25% salt, tightly packing the salted cabbage into the fermentation vessel, covering the vessel to exclude air from the cabbage, and fermenting at a temperature of 18 ◦C(Johanningsmeier et al., 2007). The aim of this work was to compare two varieties during fermentation of whole cabbage heads under optimal conditions for the spontaneous fermentation.

MATERIAL AND METHODS

White cabbage, cultivar Futoški and hybrid Bravo F1 were purchased from producers from Futog, Serbia and fermented in plastic barrels. Cleaned cabbage heads were closely placed in the barrels. Brine with dissolved NaCl was added into the barrels with cabbage heads. Overall content of added salt was 2% calculated on the weight of cabbage and brine. Fermentation process was carried out at 18^{o}C. Cabbage heads were pressed tightly and covered to prevent air from entering the cabbage mixture and CO2 from escaping from the mixture. Cabbage samples were taken from the barrel during time intervals of 20, 40, 50 and 75 days of fermentation.

The pH of the cabbage juice was measured by using a pH-meter (ExStick^{TM}, Extech Instruments, U.S.A) during fermentation. A pH meter was calibrated with pH 4 and pH 7 buffers and used for brine pH determinations. Temperature of the brine and room temperature were measured on daily basis. Total acidity, given as total lactic acid, was measured by titration using 0.1 N NaOH with phenolphthalein as indicator. Volatile acidity expressed as acetic acid was also determined. All chemical analyses were carried out in duplicate.

Fresh cabbage before fermentation process and after fermentation was sensory evaluated in order to characterize white cabbage, cultivar Futoški in relation with hybrid Bravo F1.

The four panelists were trained to evaluate fresh cabbage and sauerkraut using category scale. Fresh cabbage head shape was scored like appropriate (score 1) inappropriate (score 0) according characteristic shape of white cabbage, cultivar Futoški. Color of leaves, nervature of the leaves, leaf thickness, elasticity of the leaves, density of cabbage heads, sweetness were scored at scale of 0=not detectable to 15,5 very strong.

Fermented cabbage heads of cultivar Futoški and Hybrid Bravo F1 were also sensorial evaluated using scoring 1-5, where number 1 refers to not acceptable and number 5 to excellent taste and quality. Several commercial sauerkraut samples were evaluated by the panel during training for the purpose of selecting one that exhibited flavor attributes suitable for use as a reference. Panel was evaluated the color and uniformity of color of the pickled cabbage heads and color on the cross section, also hardness of the heads, elasticity of the leaves, presence of mucus, firmness. Odor was evaluated by intensity and note of odor. Flavour was evaluated like intensity of saltiness, sourness and note of flavour.

RESULTS AND DISCUSSION

Fig. 1 shows the decrease of pH value of white cabbage, cultivar Futoški i Hybrid Bravo brine during fermentation. Futoški cabbage achieved pH lower than 3.5 after 8 days of fermentation process, while the hybrid Bravo under same conditions achieved pH 3.5 after 11 days. Some authors cited more rapid decrease of pH in treatments with more added salt (Wiander et al., 2011).

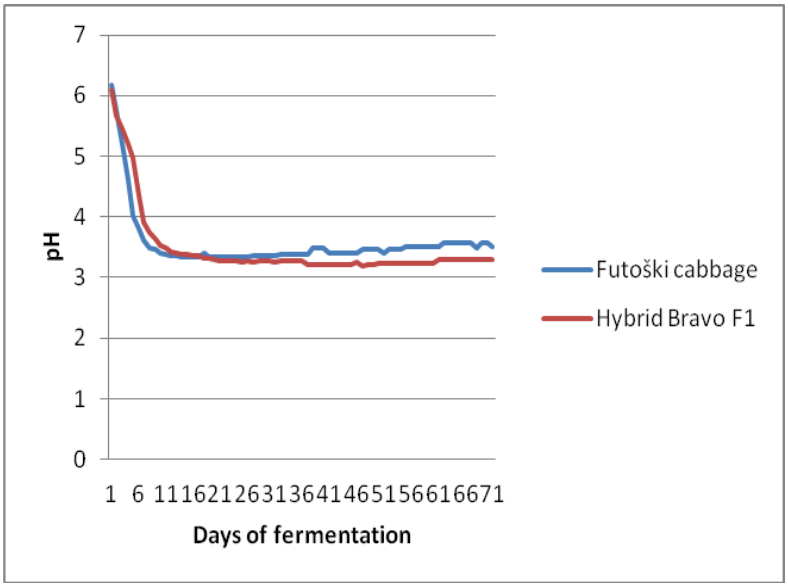

Figure 1. Decrease of pH value during fermentation process of cabbage

On Fig. 1 is shown sensorial evaluation of fresh cabbage heads of two variaties, white cabbage cultivar Futoški and Hybrid Bravo. Futoški cabbage has lighter color of the leaves, thinner leaves, milder nervature of the leaves, better elasticity of leaves. There is no significant difference between sweetness and wetness of theese two variaties.
Sensorial evalution of cabbages which were taken after 20 and 40 days of fermentation determinated that white cabbage cultivar Futoški after 20 days of fermentation achieved that diffusion of salt and brine into the cabbage tissue covered more than 90% of cabbage leaves surface. On the other hand Hybrid Bravo achived same level of diffusion only after 40 days of fermentation. This could be explained because Futoški cabbage has more loose heads and leaves are not packed so tightly as is the case with hybrid cabbage heads.

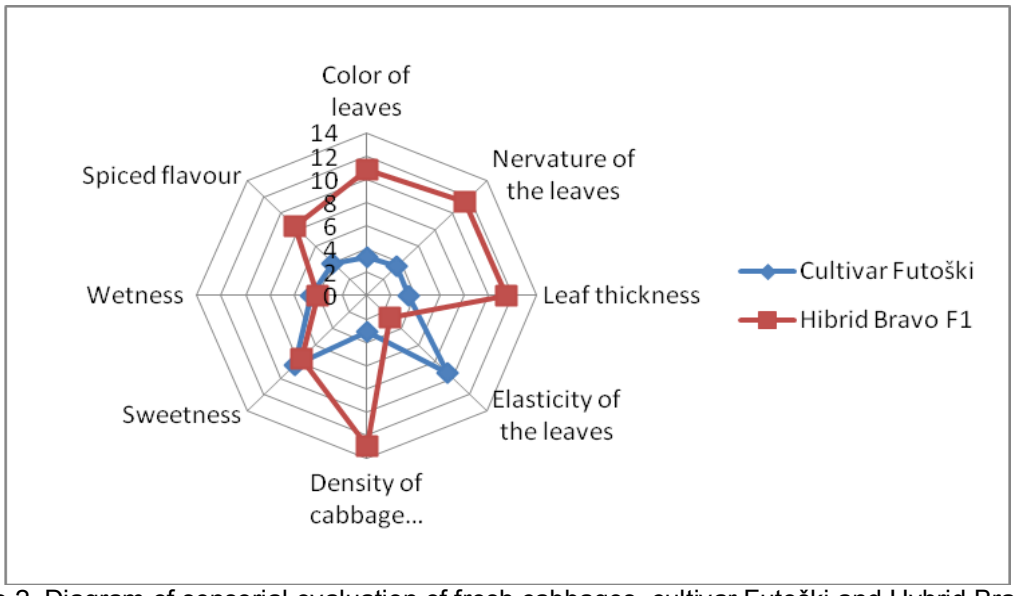

Figure 2. Diagram of sensorial evaluation of fresh cabbages, cultivar Futoški and Hybrid Bravo F1

Table 1. shows total acids content and volatile acids content produced during fermentation. Overall acidity in white cabbage, cultivar Futoški is slightly lower than the hybrid Bravo F1, but it is in the expected range according Trail et al.,1996, Pravilnik, 1979.

Table 1. Chemical characteristics of white cabbage, cultivar Futoški and hybrid Bravo F1 during fermentation

	Days of fermentation	pH	Total acids content expressed as lactic acid (%)	Volatile acids content expressed as acetic acid (%)
White cabbage, cultivar Futoški	20	3,3	1,09	0,056
	40	3,27	1,12	0,063
	50	3,28	1,13	0,065
	75	3,34	1,21	0,067
Hybrid Bravo F1	20	3,56	1,29	0,067
	40	3,21	1,36	0,075
	50	3,18	1,39	0,075
	75	3,29	1,44	0,078

Figure 2. shows diagram of sensorial evaluation of pickled cabbage heads cultivar Futoški and Hybrid Bravo F1.

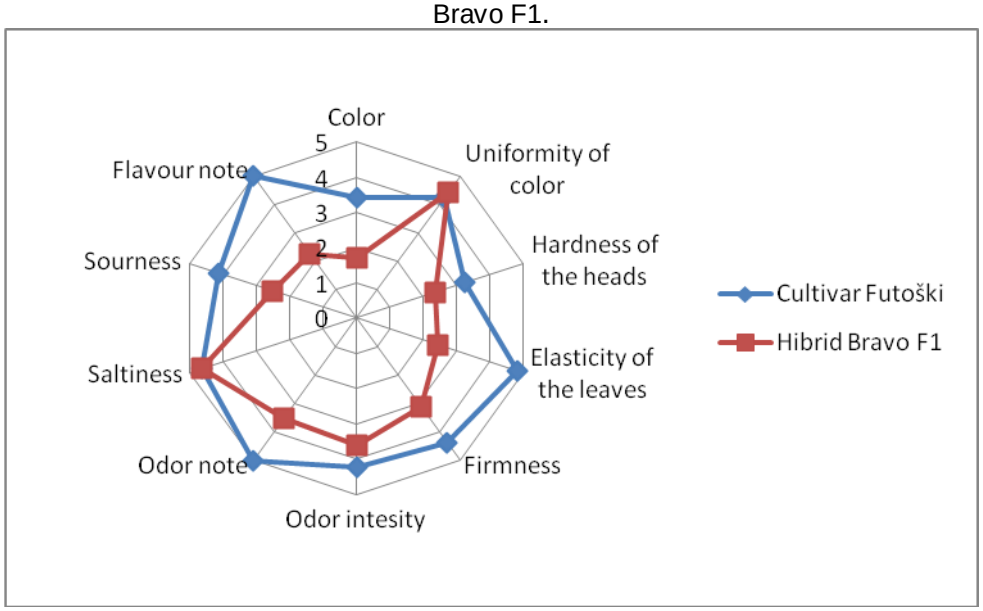

Figure 2. Diagram of sensorial evaluation of pickled cabbages, cultivar Futoški and Hybrid Bravo F1

CONCLUSIONS

Comparation of two varieties, white cabbage, cultivar Futoški and hybrid Bravo in native form and as fermented cabbage shows that white cabbage, cultivar Futoški has specific, more acceptable sensorial characteristics for fermentation. Fermentation process was developed faster in the case of Futoški cabbage, decrease of pH value was more rapid and diffusion of salted brine was more effective than in the hybrid cabbage heads. Chemical analysis shows that fermentation process was conducted in the desired direction. Slightly lower overall acidity proving mild odor and flavour in fermented Futoški cabbage what is important for the consumers who find it pleasant and acceptable.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III 046001 financed by the Ministry of Science and Technological Development, Republic of Serbia.

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PHYSICOCHEMICAL AND SENSORIAL CHARACTERIZATION OF PORTUGUESE BREAD FROM VISEU REGION

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ABSTRACT: The aim of this study was to characterise the most typical flour mixture bread (wheat flour and rye flour) produced in the Portuguese region of Viseu and evaluate if there are relevant differences between the samples tested. Nine samples from local producers were analysed considering the morphology, physicochemical (density, alveolus percentage, texture, colour and water activity) and sensorial properties. The form and dimensions of all breads were determined, as well as proximate composition and total salt content.
The forms of the breads were different, some round and others long. Nutritionally this type of bread turned out to be a food with high nutritional value, high percentage of ash (2.8-4.9%) and low values of fat, with a high range of salt content (0.4-1.4%). The physical properties were quite different between samples, although some of them are not perceptible by the consumer. This was the case of bread density whose results were 0.25-0.56 and the sensorial panel, constituted by a panel of 40 untrained tasters aged 7 to 63 years, did not find any difference. The traditional breads presented overall appreciation between 4.8 and 6.8, related with different colour and elasticity parameters.
Key words: *bread, density, texture, colour, salt, sensorial analysis*

INTRODUCTION

Bread is a food with an ancient history that assumes in diet a great cultural, social and religious importance. Bread is part of daily life for many people, starting right at breakfast, and is regarded as the basis of feed and the first source of energy (Vaz, 2004). The main ingredients of bread are flour, water, salt and yeast, of which the flour is assumed as the mass structural component, and therefore the key ingredient to obtain the bread. Water is also an essential ingredient in the formation of the mass. The salt helps in controlling fermentation, contributing to the fortification of the gluten in the flour, being decisive in the hydration of the masses, acting also as a flavour enhancer turning the bread crumb whiter (Leon and Rosell, 2007). The yeast carries out alcoholic fermentation, i.e. converts the fermentable sugars present in the mass into ethanol and carbon dioxide, which is the gas responsible for the growth of bread. Bread quality is highly dependent on the cereals most commonly used, for being considered the nobler, is wheat (*Triticum*, and more specifically *Triticum sativum*).
The advantages of a diet rich in vegetables and fibre are known since ancient times. However, only from the twentieth century there was a real concern about the low level of fibre intake in most diets. Wheat flour is an excellent source of fibre, particularly insoluble fibre (Leon and Rosell, 2007). Also the rye flour has high fibre content, especially dietary fibre, thus having positive effects on digestion and decreasing the risk of coronary heart disease, hypercholesterolemia, obesity and diabetes (Leon and Rosell, 2007). According to Pathlrana and Shahidi (2007), wheat is also a major source of antioxidants, in many cases greater than most fruits and vegetables. Among the antioxidants found in wheat highlights some phenolic acids such as ferulic, phytic or selenium acids, flavonoids, among others. As with most of the vitamins and minerals, also antioxidants are present in wheat in the bran and germ, and thus their content is reduced during the grinding process for obtaining flour (Pathlrana and Shahidi, 2007).

Addition of small amounts of rye flour to products made with wheat flour promotes the water absorption and prolongs the shelf life of the product, since it reduces the amount of amylose and amylopectin available to suffer retrogradation. As the artisan baking process is extended to industrial scale, the use of flour enhancing agents has been applied, because of the need to improve the process characteristics and shelf life of the products obtained. For decades, the enzymes were added to flour in bread production in order to improve its volume, flavour, aroma, structure of the crust and crumb, tenderness and shelf life (Nunes, 2008).
In Portugal they are known more than 100 varieties of bread. The raw material used (corn, wheat or rye) is very much dependent on the region and the weather. Various types of bread are obtained, according to cereals used (corn, wheat, rye, mixtures, whole flours), as well as the manufacturing process and type of fermentation (Almeida et al., 2008).

MATERIAL AND METHODS

Samples

In order to obtain a significant sampling, seven types of regional bread were collected in the district of Viseu, Portugal, and for each type three samples were taken triplicate. Each type of bread was accompanied by an identification form, having all the characteristics of raw materials and manufacturing process.

Chemical analyses

Several experimental determinations of chemical properties were done: moisture content, water activity, ash content, crude fat, crude fiber content, protein content, using the official methods of AOAC (2000). Determination of Chloride was done by the Mohr Method and the determination of carbohydrates was accessed by difference.

Physical analyses

In this study we performed several experimental determinations of physical nature such as size, color, density, alveolar characterization and cellular texture. To analyze the dimensions and volume of the loaves were measured the thickness, width and length. For calculating the volume the form was approximated to an ellipsoid. To determine the density were carefully cut pieces of bread in the form of parallelepipeds. From each sample were taken 15 cubes with 1 cm edge, which later were weighed on a precision scale.
In this study, the color parameters were evaluated using a colorimeter chroma meter (Minolta, Japan) expressing the results in the CIELab system coordinates: L* which is the brightness and varies between 0 (black) to 100 (white), the a* ranges from -60 (green) to +60 (red) and b* ranging between -60 (blue) to +60 (yellow). It was also determined cylindrical color coordinates: value, shade or hue (hº) and saturation (C):

$$Value = \frac{L^*}{10} \quad (1)$$

$$H^{\circ} = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad ; \quad \text{if } a^*>0 \text{ and } b^*>0 \quad (2)$$

$$C = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

To do the alveolar characterization image analysis was used, using the program "Image J", developed by Wayne Rasband at the National Institute of Mental Health United States of America. From each sample, 5 fresh slices were prepared with a thickness of 10 mm (pattern cutting). For the analysis of texture properties was used a texturometer TA-XT2 from "Stable Microsystems." The analyses were performed immediately after manufacture, and for that were cut out seven slices (10 mm) per sample, removing a cube per slice (crumb) 30 mm edge.

Sensorial analyses

Sensory analysis was performed in a laboratory prepared for that purpose, on the day of delivery of the samples by a panel of 40 untrained tasters, aged between 7 and 63 years, who were asked to rate the following attributes: crumb colour, crust colour, aroma (bread, firewood and fermented), taste (bread, wood or fermentation), elasticity, density, and finally the overall appreciation. In this test the taster expressed the intensity of each attribute through a scale where verbal Hedonic expressions are translated into numeric valuesin order to allow statistical analysis. The scale of values varied from 0 (less intense) to 10 (more intense).

RESULTS AND DISCUSSION

Tables 1. to 5. show the results of the different determinations made to all the bread samples analysed in terms of medium values, standard deviations and analysis of variance. Samples that have the same letter show no significant differences in the parameter analysed, for p> 0.05. Thus, the greater the number of letters of classification, the greater the ability to discriminate between samples.

Chemical properties

From Table 1. it is visible that the values of moisture are not much different among the samples analysed. The sample from *Flor de Cabanas* showed the highest value, although this result is not from the formulation itself, but perhaps because this sample has a high fiber content, and the presence of fibres contributes to a greater absorption of water (Cauvain and Young, 2006). On the other hand, the sample from *Viso* showed the lowest value for moisture percentage. Regarding the water activity of the bread samples, and although the values were not so different from each other, the truth is that statistically there are some differences to be noticed.

In relation to the ash content, the samples from *Oliveira & Alves*, *Panifil*, *Pedregal* and *Flor de Cabanas*, showed very similar values (from 3.86 to 3.96), not statistically different as the analysis of variance confirmed. The sample showing a higher percentage of ashes is the sample from *Pazurara*, being this value statistically different from all the others, and this could be explained by the fact that this sample has in its constitution flour 130, with high power extraction.

Table 1. Chemical composition of the bread samples analysed.

Sample	Moisture (%)	Ash (%)	Fat (%)	Fiber (%)	Protein (%)	Salt (%)	Carbo-hydrates (%)	Aw
F. Car.	47.42±0.14^{b}	3.80±0.01^{c}	0.51±0.11^{b}	0.06±0.01^{e}	14.59±0.25^{c}	0.40±0.02^{e}	81.1±0.30^{b}	0.96±0.77^{b}
F. Cab.	53.59±0.16^{a}	3.86±0.11^{cd}	0.50±0.12^{bc}	0.33±0.03^{d}	14.45±0.39^{c}	0.96±0.02^{f}	81.2±0.24^{a}	0.95±0.50^{b}
Pazurara	35.27±0.20^{e}	4.90±0.03^{a}	0.26±0.02^{c}	0.05±0.01^{e}	10.23±0.35^{e}	1.06±0.03^{c}	84.7±0.29^{e}	0.94±0.64^{ac}
Ol.&Alves	37.21±0.26^{d}	3.96±0.02^{d}	0.31±0.02^{bc}	0.55±0.01^{b}	11.25±0.24^{d}	1.47±0.03^{a}	84.8±0.22^{d}	0.94±0.58^{cd}
Panifil	36.70±0.28^{d}	3.94±0.01^{d}	0.37±0.09^{bc}	0.18±0.01^{c}	12.90±0.21^{a}	0.93±0.04^{f}	82.8±0.25^{d}	0.95±0.57^{ab}
Viso	28.08±0.14^{c}	2.76±0.01^{b}	0.37±0.08^{bc}	0.29±0.02^{d}	8.92±0.19^{b}	0.74±0.02^{d}	88.0±0.17^{c}	0.93±0.42^{d}
Pedregal	35.69±0.16^{e}	3.88±0.01^{d}	1.19±0.09^{a}	0.89±0.01^{a}	10.52±0.21^{de}	1.14±0.03^{b}	84.4±0.23^{e}	0.94±0.33^{cd}

Results are given as mean ± standard deviation (n = 3). Samples in the same column with the same letter are not statistically different for p>0.05.

In terms of fat content, the samples from *Flor de Cabanas*, *Oliveira & Alves*, *Panifil* and *Viso*, showed values statistically similar to each other. On the other hand, the sample from *Pedregal* contrasts with the others, showing a much higher value, which might be explained by the addition of any kind of lipidic ingredient, although this information was not provided by the manufacturer.

The levels found for cellulose are similar in the samples from *Flor de Carregal* and *Pazurara* as well as between in those from *Flor de Cabanas* and *Viso*. The sample showing the

highest fibre content is the sample from *Pedregal*, perhaps because in its constitution has two types of flour with high power extraction, wheat 130 and rye 150, as stated in the ingredients form.
The amounts of protein in the different samples analysed are in general different from each other, except for samples from *Flor de Cabanas* and *Flor de Carregal*, which have higher protein contents.
The results in Table. 4 also show that the salt contents in the samples analysed are quite different, with sample from *Oliveira & Alves* presenting the higher chloride percentage. Considering the information on the identification form for that bread, according to which the salt content should be lower, this fact may be due to the addition of a higher quantity of salt than that established in the formulation.
Regarding the contents of carbohydrates, the results among the samples do not differ significantly. Even though, the sample from *Viso* stands as the one with the higher value, which can be explained by its lower values in terms of ash and protein contents.

Physical properties
According to the values shown in Table 2. in terms of the colour coordinates for the crust, it was found that the parameters L and Value do not differ significantly. These parameters indicate that the samples were quite dark (with values standing near the middle of the scale). Regarding the parameter a in the samples *Flor de Carregal*, *Pazurara* and *Viso*, the values were similar to each other, as well as in the samples from *Flor de Cabanas* and *Panifil*. These values indicate the predominance of the red colour over green. With relation to the parameters b, hue (cylindrical coordinates colour) and c (saturation or chroma), no significant differences were seen between the samples. Furthermore, and since the values of b are highly positive, the colour yellow is dominant over blue, as expected. The results for the crumbs are quite similar, although showing a lighter colour and less intense brown.

Table 2. Colour properties of the bread samples analyzed.

Crust						
Sample	**L**	**a**	**b**	**Hue (º)**	**C**	**Value**
F. Car.	59.75±2.69^{b}	10.78±1.70b^{c}	30.02±2.92^{bc}	70.30±2.28^{c}	31.92±3.20^{abd}	5.98±0.27^{b}
F. Cab.	50.38±6.91^{a}	15.71±3.07^{a}	30.38±5.37^{bc}	62.45±5.53^{b}	34.34±5.48^{d}	5.04±0.70^{a}
Pazurara	60.46±6.33^{b}	10.82±2.62b^{c}	27.03±3.17^{a}	68.47±2.87^{c}	29.15±3.91^{c}	6.05±0.64^{b}
Ol.&Alves	58.96±3.42^{b}	10.08±2.63^{c}	28.01±3.15^{ac}	70.35±4.00^{c}	29.84±3.62^{bc}	5.90±0.35^{b}
Panifil	52.82±7.61^{a}	14.40±2.88^{a}	31.11±2.85^{bc}	65.27±4.54^{a}	34.38±3.19^{d}	5.28±0.77^{a}
Viso	57.11±2.60^{b}	10.61±1.47^{bc}	28.2±2.06^{ac}	69.44±1.94^{c}	30.15±2.37^{abc}	5.71±0.26^{b}
Pedregal	57.25±3.96^{b}	12.15±1.68^{b}	30.42±1.53^{bc}	68.30±2.55^{c}	32.79±1.83^{ad}	5.73±0.40^{b}
Crumbs						
Sample	**L**	**a**	**b**	**Hue (º)**	**C**	**Value**
F. Car.	66.69±2.55^{c}	3.11±0.29^{c}	19.95±0.97^{d}	81.16±0.52^{b}	20.19±1.01^{d}	6.67±0.26^{c}
F. Cab.	68.75±3.81^{c}	1.57±0.34^{d}	13.21±1.51^{a}	83.29±0.92^{f}	13.30±1.56^{a}	6.88±0.39^{c}
Pazurara	68.96±1.93^{c}	1.47±0.23^{d}	16.19±1.15^{c}	84.84±0.65^{a}	16.26±1.17^{c}	6.90±0.20^{c}
Ol.&Alves	59.65±2.92^{ab}	4.03±0.47^{b}	19.34±1.13^{d}	78.26±0.94^{d}	19.76±1.20^{d}	5.96±0.30^{ab}
Panifil	68.37±3.25^{c}	2.13±0.47^{a}	17.22±1.26^{b}	83.02±1.15^{f}	17.35±1.32^{b}	6.84±0.33^{c}
Viso	61.34±4.21^{ab}	4.30±0.36^{b}	19.35±1.20^{d}	77.46±0.79^{e}	19.83±1.24^{d}	6.13±0.43^{ab}
Pedregal	58.67±2.19^{b}	3.13±0.24^{c}	16.68±0.70^{bc}	79.37±0.58^{c}	19.98±0.73^{bc}	5.87±0.22^{b}

Results are given as mean ± standard deviation (n = 30). Samples in the same column with the same letter are not statistically different for p>0.05.

The larger sample (Table 3.) was the *Panifil*, which is distinguishable from the others, perhaps because it has a higher content of salt and proteins, thus inducing a good fermentation. As to the crumbs density, the sample from Flor de Cabanas is highlighted as the denser, while all others are statistically identical. As to the alveolar characterization, the

sample from *Viso* revealed a greater total area, a higher alveolar percentage as well as a bigger alveolus average size.

Table 3. Physical properties of the bread samples analyzed.

Sample	Whole bread volume (cm^3)	Crumbs density (g/cm^3)	Slice alveolar characterization			
			Area ($pixel^2$)	Nº Alveolus	% Alveolar	Size ($pixel^2$)
F. Car.	697.00±7.28^{c}	0.37±0.06^{bc}	1930258.2±430765.3^{b}	405.4±74.1^{a}	23.8±4.7^{bc}	4764.8±565.7^{b}
F. Cab.	565.42±7.37^{f}	0.56±0.06^{a}	1185786.8±769930.5^{bc}	254.6±129.7^{c}	17.4±12.7^{bc}	4684.0±1399.1^{b}
Pazurara	737.23±5.76^{b}	0.38±0.05^{b}	721473.8±124114.1^{c}	107.2±15.4^{b}	10.4±1.8^{b}	6839.6±1383.8^{b}
Ol.&Alves	474.89±8.99^{e}	0.25±0.03^{e}	1319942.8±412921.5^{bc}	231.8±50.6^{bc}	25.1±8.1^{c}	5660.2±925.0^{b}
Panifil	878.16±6.87^{a}	0.31±0.04^{d}	1178604.8±417663.8^{bc}	186.4±63.7^{bc}	14.2±5.1^{bc}	6567.0±1911.5^{b}
Viso	632.64±7.73^{d}	0.25±0.02^{e}	3559231.2±525330.7^{a}	229.2±24.8^{bc}	45.3±5.6^{a}	15753.2±3299.5^{a}
Pedregal	548.49±8.87^{f}	0.32±0.05^{cd}	1500673.4±4078623.0^{bc}	274.0±48.7^{ac}	24.3±5.5^{c}	5451.2±874.5^{b}

Results are given as mean ± standard deviation (n = 3 for volume, n = 15 for density, n = 5 for alveolar characterization). Samples in the same column with the same letter are not statistically different for p>0.05.

By analyzing the results of textural properties in Table 4., it was found that in general the samples are very similar. The parameters chewiness and hardness in the sample from *Flor de Cabanas* are higher, which might be explained by the higher density as previously reported. In terms of cohesiveness and elasticity, it was the sample from *Panifil* that showed the highest values.

Table 4. Textural properties of the bread samples analyzed.

Sample	Hardness (N)	Elasticity (%)	Cohesiveness (dimensionless)	Chewiness (N)
F. Car.	5.52±1.53^{b}	88.92±2.72^{c}	0.60±0.05^{a}	2.91±0.67^{b}
F. Cab.	9.06±1.94^{a}	91.94±1.36^{abc}	0.72±0.03^{c}	6.02±1.27^{a}
Pazurara	4.71±0.90^{b}	90.56±2.80^{bc}	0.75±0.04^{bc}	3.19±0.48^{b}
Ol.&Alves	1.94±0.63^{c}	94.15±2.55^{abd}	0.75±0.03^{bc}	1.35±0.39^{c}
Panifil	1.59±0.34^{c}	96.25±2.90^{d}	0.79±0.02^{b}	1.21±0.27^{c}
Viso	2.05±0.60^{c}	95.31±3.06^{ad}	0.73±0.04^{bc}	1.41±0.32^{c}
Pedregal	2.42±0.82^{c}	96.14±1.63^{d}	0.78±0.02^{bc}	1.79±0.50^{c}

Results are given as mean ± standard deviation (n = 7). Samples in the same column with the same letter are not statistically different for p>0.05.

Sensorial properties

According to what is described in Table 5., it was found that the different samples were equally perceived by the tasters in relation to the following attributes: bread aroma, wood aroma and ferment aroma, as well as in terms of density, wood flavour and ferment flavour. Regarding the sensory parameters: crust colour, crumb colour, elasticity and ferment flavour, the perceptions were also very similar. As regards the overall assessment, the samples were also perceived in a similar way, highlighting however the sample from *Flor de Cabanas*.

Table 5. Results of the sensorial analysis.

Sample	Bread aroma	Wood aroma	Ferment aroma	Crust colour	Crumbs colour	Density
F. Car.	5.00±1.9^{a}	4.05±2.3^{a}	4.63±2.5^{a}	5.31±1.8^{bc}	5.69±1.7^{ab}	5.83±1.8^{a}
F. Cab.	5.35±2.2^{a}	3.29±2.2^{a}	4.42±2.4^{b}	4.38±2.4^{b}	3.68±2.4^{cd}	4.57±2.2^{a}
Pazurara	5.35±2.2^{a}	3.29±2.2^{a}	4.42±2.4^{a}	4.38±2.4^{b}	3.68±2.4^{cd}	4.57±2.2^{a}
Ol.&Alves	5.14±2.0^{a}	4.00±2.6^{a}	3.37±2.4^{a}	6.80±1.8^{a}	3.37±1.8^{cd}	4.52±2.1^{a}
Panifil	6.03±1.7^{a}	4.53±2.5^{a}	4.13±2.0^{a}	6.41±2.0^{a}	6.17±1.8^{b}	4.9±2.0^{a}
Viso	5.91±1.6^{a}	4.54±2.6^{a}	4.76±2.5^{a}	5.19±2.3^{bc}	4.53±1.9^{ac}	4.55±2.5^{a}
Pedregal	5.79±2.0^{a}	3.31±2.3^{a}	3.97±2.6^{a}	5.70±1.6^{ac}	6.80±1.9^{b}	4.69±2.1^{a}

Sample	Elasticity	Ferment taste	Wood taste	Bread taste	Global appreciation
F. Car.	5.12±2.0^{abc}	4.64±2.4^{b}	3.94±2.5^{a}	5.00±2.4^{a}	5.22±2.1^{ab}
F. Cab.	5.99±1.9^{ab}	4.43±2,7^{b}	3.014±2.3^{a}	5.23±2.4^{a}	6.95±2.1^{a}
Pazurara	5.99±1.9^{ab}	4.43±2.7^{b}	3.01±2.3^{a}	5.32±2.4^{a}	6.59±2.1^{a}
Ol.&Alves	4,33±2.2^{c}	2.75±1.9^{a}	3.92±2.5^{a}	4.88±2.2^{a}	4.79±2.6^{b}
Panifil	5.53±2.0^{abc}	4.11±2.1^{ab}	4.12±2.6^{a}	5.52±1.8^{a}	6.02±1.8^{ab}
Viso	6.15±2.2^{a}	3.68±2.5^{ab}	4.09±2.7^{a}	5.19±2.4^{a}	5.74±2.1^{ab}
Pedregal	4.84±2.3^{abc}	3.42±2.2^{ab}	3.34±2.4^{a}	5.02±2.2^{a}	5.48±2.4^{ab}

CONCLUSIONS

The results from this work showed that the breads were different in shape and size, as well as density or alveolar characterization. Nutritionally this type of bread turned out to be a good food with high percentage of minerals and low values of fat. However, the salt content varied quite a lot among samples. The physical properties were quite different between samples, although some of them were not perceptible by the panel members. This happened, for example with density, whose results varied a lot and the sensorial panel was not able to differentiate the samples. The traditional breads presented a global appreciation between 4.8 and 6.8, related with different colour and elasticity parameters.

ACKNOWLEDGEMENTS

The authors thank the financial support to the research centre CI&DETS (IPV - Viseu).

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ANTIOXIDANT CAPACITY AND SENSORY CHARACTERISTICS OF SPECIAL HERB BRANDY

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ABSTRACT: Plum brandy is traditional Serbian alcoholic beverage, made by distillation of fermented plum variety *Prunus domestica.* Distillate is colorless and has characteristic fruit aroma, which originates from the component of the inner layer of the plum skin. According to the Serbian tradition, plum brandy is sometimes mixed with different medicinal herbs, and these products are treated as forms of herbal medicine. Medicinal herbs are rich source of polyphenols and other valuable compounds, which can contribute to the functional properties of brandies. These products are usually produced from a large number of herbs and spices, which, also, change the aroma complex and contribute to the color. In this study, it was investigated the effects of two herbal compositions which were consisted of 44 plants with different ratio of aromatic and bitter herbs on sensory characteristics and antioxidant capacity of herbal brandies. Sensory assessment of samples was performed by using modified Buxbaum model of positive ranking. The total phenolic content of samples was determined according to the Folin-Ciocalteu spectrophotometric method. The antioxidant capacity (ATC) was tested by using two methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing ability of plasma (FRAP). The total sensory quality of samples was 17.85 and 17.95, which were very good scores. The total phenolic content of samples was 145.56 and 130 mg/L gallic acid equivalents, while the antioxidant capacity was 0.69 and 0.61 mM Trolox according to DPPH assay, and 2.08 and 1.75 FRAP units according to FRAP method. The obtained results suggested that higher content of the bitter herb significantly influenced the antioxidant capacity and not significantly affected the sensory profile of special brandy. Based on sensory assessments, the sensory properties of these special brandies were completely acceptable for the assessors.
Key words: *herb brandy, sensory characteristics, antioxidant capacity*

INTRODUCTION

Official Gazette of the Republic of Serbia for regulation of spirits appoints that herb brandy has to be made from distillate or mixture of distillate of agricultural origin and macerate and/or distillate of macerate of aromatic plants (parts or whole plant). The ethanol content of basic distillate must have at least 37.5 % (v/v) (Pravilnik o kategorijama, kvalitetu i deklarisanje rakije i drugih alkoholnih pića, 2010).

Herb brandies are produced in many Mediterranean countries and in a number of Balkan countries, such as Serbia, Macedonia, Bulgaria, Greece and Croatia. The distillate for production herb brandy is usually made from various fermented fruits depending on the production's tradition and the region of origin. The most used distillate for production Serbian herb brandy is made by double distillation of fermented plum, variety *Prunus domestica.* Distillate is colorless and has characteristic fruit aroma, which originates from the component of the inner layer of the plum skin (Popović et al., 2008).

Serbia has a century old tradition of collecting and using aromatic and medical herb. The wisdom of using medicinal herb to promote good health and treatment various diseases have been saved to present days. According to this heritage, distillate is mixed with different medicinal herbs, and these products are still treated as forms of folk medicine. The beverage is usually produced from a large number of herbs and spices, which also change the aroma complex and contribute to the color.

In production of beverage with herb aroma, the most frequently used method for extraction the aromatic and active phytochemical compounds from plants is maceration in the ethanol/ water solution. This process has benefits compared to other separation techniques. Processing on low temperature does not provoke denaturation of herb components and preserves the health properties of beverage (Tonutti and Liddle, 2010). Herbs are rich source of bioactive components like different polyphenols, phytoalexins, alkoids, cumarins, diterpenes, lignans, organic acids, organic oils, volatile oils, etc (Saroya, 2011). Medicinal herb can have many health benefits for consumers including antioxidant activity, digestive stimulation action, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic effects and anticancerogenic potential (Wojdylo et al., 2007). In the last decade the antioxidant characteristics of beverages have become very important for its quality (Gorjanović et al., 2010).
The functional and sensory characteristics of food and beverage depend of the concentration, combination and chemical composition of various herbs. The variation and diversity of chemical composition of plants depends on various effects, such as the anatomical part of plant, the location and time of harvesting, the storage condition and many others. To produce the beverage with standard chemical composition and quality, the use of herbal extracted is the best solution.
In this study, it was investigated the effects of two herbal composition on sensory characteristics and antioxidant capacity of herb brandies. Both compositions were produced with 44 different plants but with different ratio of bitter herbs.

MATERIAL AND METHOD

The plum brandy was obtained from local homemade manufacture from the region of Kruševac and herbs from local health food store. Air-dried plant or its parts were cut into small pieces and added in 45 % v/v alcohol medium (plum brandy). The extraction of plants (two plant composition with different concentrations of bitter herbs) was carried out in glass bottles which were placed on shaker in dark place at room temperature for period of 10 days. The mixture was filtered through 70 g/m^3 filter paper.
In study it was analysed two different herb brandies with various ratios of bitter herbs. Herb brandies contain the flowing extracts of 44 plants (39 herbs (aromatic and bitter), four dried fruits and wood's bark): sweet odorata (*Asperula odorata*), peppermint (*Mentha piperita* L.), mountain germander (*Teucrium montanum* L.), wall germander (*Teurium chamaedrys*), hibiscus (*Hawaiian hibiscus*), stinging nettle (*Utrica dioica* L.), common sage (Salvia officinalis L.), pot marigold or english marigold (*Calendula officinalis* L.), camomile (*Matricaria chamomilla* L.), mellisa (*Melissa officinalis* L.), sweet flag (*Acorus calamus*), wild thyme or creeping thyme (*Thymus serpyllum* L.), horehound (*Marrubium vulgare*), hawthorn (*Crataegus oxyacantha* L.), elder or elderberry (*Sambucus nigra* L.), common gypsy weed or herbal speedwell (*Veronica officinalis*), common yarrow (*Achillea millefolium* L.), sweet marjoram (*Origanum majorana* L.), coltsfoot (*Tussilago farfara* L.), grand wormwood (*Artemisia absinthium* L.), cypress spurge (*Euphorbia cyparissias* L.), horsetail (*Equisetum arvense* L.), common juniper (*Juniperus communis* L.), herb hyssop (Hyssopus *officinalis*), rosemary (*Rosmarinus officinalis* L.), gentiane (*Gentiane lutea*), blueberry (*Vaccinium mytillus*), european mistletoe or common mistletoe (*Viscum album* L.), shepherd's purse (*Capsella bursa pastoris* L.), linnean herbarium (*Paris quadrifolia* L.), centaury (*Erythraea centaurium* Pers.), fennel (*Foeniculum vulgare* Mill.), elecampane or horse-heal (*Inula helenium*), common chicory (*Cichorium intybus*), anise (*Pimpinella anisum* L.), ribwort plantain (*Plantago lanceolata*), vanilla (*Vanilla planifolia*), cinnamon (*Cinnamomum* div.), eugenia (*Eugenia caryophyllata* L.), common fig (*Ficus carica* L.), grape (*Vitis vinifera* L.), plum (*Prunus domestica* L.), apple (*Pirus malus* L), oak wood (*Quercus* sp.).
The total phenolic content (TPC) of herb brandies samples were determined according to the Folin-Ciocalteu spectrophotometric method described by Singleton and Rossi (1965). The total antioxidant capacity was evaluated using DPPH (Kaneda et al., 1995) and FRAP methods (Benzie and Strain, 1996).

Sensory characteristics of the brandies enriched with aromatic and bitter herbs were determined using modified Buxbaum model of positive ranking (Tešević et al., 2005). The common quality parameters were evaluated: clearness – parameter which contributes to the overall visual liking of drink which depend on the purity and hue of color (max 1 points); color - visual impression created as a result of irritation of the retina of the eye with light rays of different wavelengths (max 1 points); distinction – parameter which clearly defines specific and distinctive characteristic synonymous for certain categories of strong alcoholic beverages (max 2 points); odor - a sensory attribute resulting from stimulation of the olfactory receptors in the nasal cavity by certain volatile substances (max 6 points); taste - a sensory attribute resulting from stimulation of the gustatory receptors in the oral cavity by certain soluble substances (max 10 points) (Nikićević, 2005). In this evaluation a brandy sample may have a maximal score of 20 points. Sensory tests were carried out using a panel of five qualified assessors (experts). Sample were served and assessed at professional testing room (air temperature 20 °C, adequate daylight, peace and quietness).
The experimental data were subjected to an analysis of variance (ANOVA), while Tuckey's test was calculated to detect difference ($p \leq 0.01$) between the mean values. Statistical analyses were performed with the statistical program MS Excel (Microsoft Office 2007 Professional).

RESULTS AND DISCUSSION

The first goal of this study was to examine the effect of two various concentrations of bitter herbs on sensory and antioxidant characteristics of herb brandies. The TPC and antioxidant potential were presented in Table 1. The results of ANOVA showed that the various concentration of bitter herbs very significantly influence on the TPC and antioxidant characteristic (ATC) of these herb brandies ($p < 0.01$). Based on these results, the adding the herb in spirit significantly influence on the TPC and increase the ATC of plum distillate. The concentration of herbs is also important factor for the TPC and ATC, the higher amount of herb increase ATC and improves the healthy properties.

Table 1. TPC and antioxidant capacity of herb brandies

sample	TPC (mg/L GAE)	FRAP (mmol TE)	DPPH (mmol TE)
P	5.10 ± 0.25^{A}	0.12 ± 0.02^{A}	0.050 ± 0.002^{A}
HB1	145.56 ± 0.00^{B}	1.75 ± 0.03^{B}	0.610 ± 0.010^{B}
HB2	130.00 ± 2.56^{C}	2.08 ± 0.03^{C}	0.691 ± 0.015^{C}

Each value is the mean ± standard deviation of three replicate experiments.
Within each column the different letters mean that differences were very significant according to Tuckey's HSD test at $\alpha \leq 0.001$

a. Total phenol content, expressed as milligram of gallic acid equivalents per liter of brandy.
b. Total antioxidant capacity expressed as mmol of Trolox equivalent.

P - plum brandy; **HB1 -** herb brandy with higher content of bitter herb; **HB2** - herb brandy with lower content of bitter herb.

The sensory scores of evaluated herb brandy samples were 17.85 and 17.95, which were a very good score and higher value than sensory score for basic alcohol medium (15.8). The herbal components significantly affected on the sensory characteristics of plum distillate, and gave the specific herb aroma to the samples. The sample HB1 had more insensitive bitter taste then HB2, due to higher amounts of bitter herbs. The sensory assessors evaluated both samples with very similar value; therefore higher bitterness was acceptable for the assessors. The color of herb brandy samples had characteristic green tones, which originate from herb components and change the color of otherwise colorless distillate.

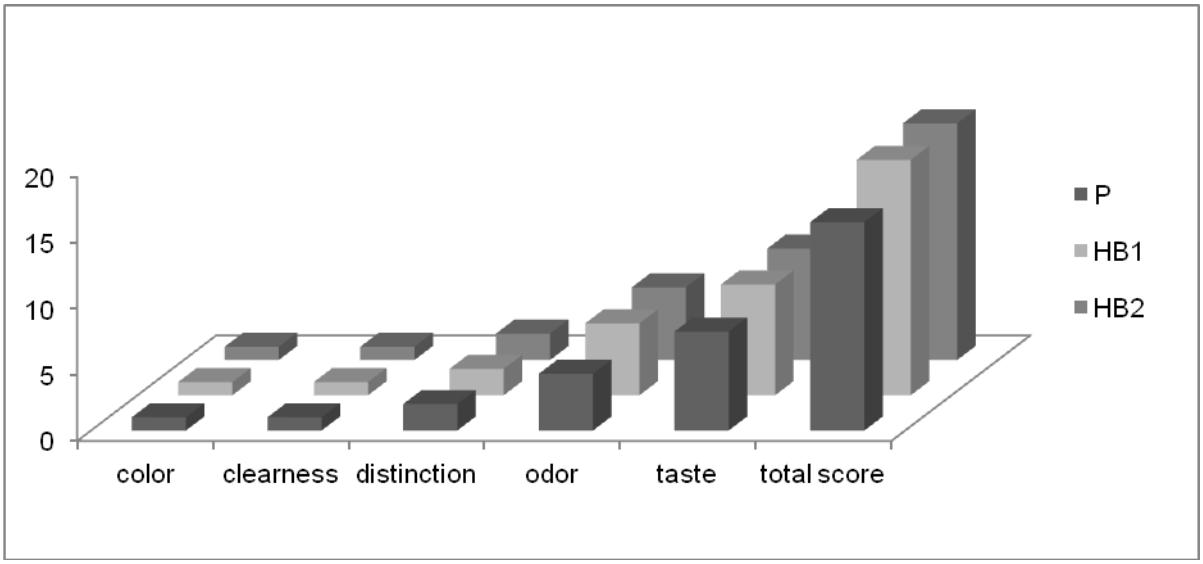

Figure1. Sensory characteristics of herb brandies

CONCLUSIONS

The present study showed that the higher quantity of bitter herbs in the plant mixture of samples significantly affect on the TPC and ATC. The sensory characteristics of herb brandy samples were changed, but not significantly affect on the total sensory scores. The both bitterness intensity of the samples was acceptable for the assessors. Based on these results, it was demonstrated that extracting the higher amount of bitter herb increased the ATC, and did not significant effect on the sensory score. The used herb improves the sensory characteristics and improved healthy properties of the spirits. According to these results, the sensory properties of special brandies were completely acceptable for the assessors.

ACKNOWLEDGEMENTS

This paper is a result of the research within the projects 46001/46010, financed by the Ministry of Education and Science, Republic of Serbia.

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PREDICTION OF SOME OENOLOGICAL CHARACTERISTICS OF WINE PROŠEK USING NEAR INFRARED SPECTROSCOPY

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ABSTRACT: The aim of this study was to examine the possibility to predict concentrations of ethanol, fructose, glucose and total acidity in wine Prošek using specific wavelength.
Wines Prošek were produced using two grape varieties (Plavac mali and Pošip). Physical-chemical measurements (relative density, real alcohol, total extract, reducing sugars, pH, total acidity, volatile acidity, ash, free sulphur dioxide, total sulphur dioxide) were conducted for wine samples. The absorbance of wines Prošek was measured using defined wavelengths with the instrument NIR-128-1.7-USB/6.25/50 µm with scan range 904-1699 nm.
Calibration equations were developed from NIR data using partial least squares (PLS) regression with internal cross validation. Using PLS regression, very good calibration statistics (R^2 > 0.80) were obtained for the prediction of total alcohol, fructose, glucose and acidity for the red wine Prošek produced from grape variety Plavac mali regardless which yeast was used in the production. The same efficiency was detected for the white wine Prošek produced from the grape variety Pošip only in predicting content of alcohol and acidity. From the stacking data of the experiment and gained from the models can be concluded that near infrared spectroscopy could be used as rapid alternative method for the prediction of the concentration of alcohol and acidity for Prošek wines regardless the grape variety and in prediction of concentration of fructose and glucose just for Prošek wines produced from grape variety Plavac mali regardless the yeast used in the wine production.
Key words: *NIRs, wine Prošek, prediction*

INTRODUCTION

Growing interest in the safety and traceability of food and beverages is a challenge for the development of methods for proving and detection. Methods that require pre-treatments of the sample also must include highly-skilled personnel what is the main reason why fast and non-destructive methods are more and more in use (Alishahi et al., 2011). Among them, near infrared spectroscopy has proven to be a successful analytical method for analysis of a variety of food products (Nicolaï et al., 2007; Reid et al., 2005; Sirisomboon et al., 2007). NIR region (750 – 2500 nm) is very useful spectral fingerprint of food samples in which vibration and overtone combinations of basic bonds (O–H, C–H and N–H) are the main recordable phenomena (Williams et al., 2001). In the food industry, NIR measurements are used in measurement (Hernández Gómez et al., 2006; Liu et al., 2010) evaluation of quality (Sirisomboon et al, 2007), quality prediction (Cayuela et al., 2010), and differentiation of samples (Reid et al, 2005) and prediction (Ozaki et al., 2007). Previous studies have shown that some oenological parameters for red wine can be predicted using of near infrared spectroscopy (NIRs). Wine is an important component in Mediterranean dietary tradition and is considered as an important health benefactor since it is rich with antioxidant compounds. The antioxidant capacity of wine (related with phenolic compounds) depends on the grape variety, vineyard location, cultivation system, climate, soil type, vine cultivation practices, harvesting time, production process and ageing (Shahidi and Naczk, 1995) and grape maturity (Le Moigne et al., 2008). The consumer is relating wine quality with the name and

origin, expecting uniformity what is often not easy for the producer regarding different factors influencing the harvest (precipitation, number of sunny days, temperatures, etc.).
This paper discusses the ability of NIRS in prediction of concentrations of ethanol, fructose, glucose and total acidity in wine Prošek using specific wavelength. The inter-relationships between chemical properties and sensorial evaluation can provide reliable results (Williams et al., 1988; Bueno et al., 2010) that could be used in the production and market positioning of the wine (Parpinello et al., 2009; Charters & Pettigrew, 2007).

MATERIAL AND METHODS

Wine production

Dessert wines "Prošek", were produced by semi scaled fermentation from dried grapes of cv. Pošip and *Plavac mali* in the harvest of 2008. Grapes were dried in the glasshouse for 5 days and 18 days, respectively. Dried grapes were crushed, destemmed and sulphited with potassium metabisulphite (15 g/hL). After 4 hours of skin contact at 15 °C, the Pošip must was racked into a 25-litre glass container. Alcoholic fermentation was done with selected yeast Fermol Cryoaromae (*Saccharomyces cerevisiae var. uvarum*, 30 g/hL, AEB s.p.a., Brescia, Italy). Alcoholic fermentations for Plavac mali cv. were done using comercial yeasts (Lalvin EC 1118, *Saccharomyces cerevisiae var. bayanus*, 40 g/hL, Danstar ferment, Switzerland; and Fermol Cryoaromae, *Saccharomyces cerevisiae var. uvarum*, 30 g/hL, AEB s.p.a., Brescia, Italy) and with native yeasts. Maceration for cv. Plavac mali took 5 days and the pomace was punched down twice daily, after which it was pressed on the hydraulic press (pressure <2 bar). The must was put in 10-litre glass vials, where the alcoholic fermentation continued. Temperature during fermentation was between 22 and 24 °C. The first racking was done 29 days, and the second 184 days (6 months) after the beginning of fermentation. After the second racking, Prošek was bottled. All experiments were performed in duplicate.

NIR analysis

NIR spectra of Prošek wine samples were collected in the range of 904–1699 nm using a Control Development, Inc., NIR-128-1.7-USB/6.25/50 µm, with installed Control Development software Spec32. NIR spectroscopy is based on the electromagnetic absorption at the near-infrared region but the spectral analysis has to be assisted with various chemometric techniques (Ding & Xu, 1999; Alishahi et al., 2010).

Application of NIR to fermentation process - wine

NIR has been applied to the measurement of the concentrations of some constituents in wine. Previous studies have measured concentration of ethanol, fructose, and tartaric acid in white and red wines using NIR, based on optical density (transmittance) of wine samples in the wavelength region of 800 – 2400 nm. Six wavelengths were found to be most characteristic for ethanol, fructose and tartaric acid: 1072, 1450, 1696, 1816, 2150 and 2232 nm. Applied NIR instrument collected data in the range of 904 – 1699 nm, and for the prediction were used 3 specific wavelengths of the absorbance spectrum (1072, 1450, 1696 nm) in prediction of ethanol, fructose, glucose and total acidity in wine Prošek. Data were analysed using program *Statistica v. 8*.

RESULTS AND DISCUSSION

Wine Prošek were produced using two grape varieties (Plavac mali and Pošip). Physical-chemical measurements (relative density, real alcohol, total extract, reducing sugars, pH, total acidity, volatile acidity, ash, free sulphur dioxide, total sulphur dioxide) were conducted for wine samples.

Table 1. Average values for basic oenological parameters of dessert wine Prošek, from Pošip and Plavac mali cv., vintage 2008

Physical-chemical parameters	Grape varieties			
	Plavac mali			Pošip
	EC 1118	Cryoaromae	Native	Cryoaromae
Relative density (20/20ºC)	27.8	25.5	26.3	27.0
Real alcohol (vol %)	14.3	13.4	13.7	10.5
Total extract (g/L)	254.5	261.3	259.5	334.2
Reducing sugars (g/L)	215.2	206.6	214.5	280.7
pH	3.8	3.7	3.8	3.9
Total acidity (g/L)	7.7	7.9	6.8	7.4
Volatile acidity (g/L)	1.6	1.9	1.5	1.9
Ash (g/L)	3.8	3.9	3.8	5.2
Free sulphur dioxide (mg/L)	3	4	2	11
Total sulphur dioxide (mg/L)	37	36.5	36.5	125.5
Glucose (g/L)	69	81	84	210
Fructose (g/L)	180	137	145	166

Table 1 presents results of oenological parameters of wines produced from different grape varieties, with different yeasts. Using NIR process analyser, each spectrum of different wine Prošek from cv. Pošip and Plavac mali was recorded. Each sample was recorded in triplicate and the mean value was calculated. In figure 1 are presented means of NIR spectrums (absorbance).

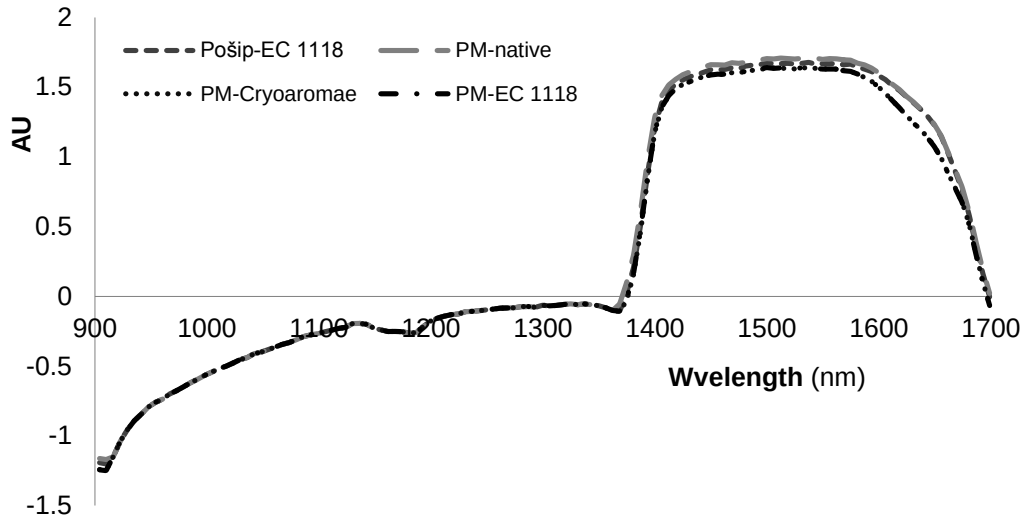

Figure 1. Absorbance spectra for wine Prošek produced from different grape (Plavac mali (PM), and Pošip)

Analysing presented spectres, it seems that all records are very similar, regardless of the grape used in the wine production, with an exception in the range 1400-1700 nm, where the curves show slight differences. Presented results are in accordance with the study conducted by Cozzolino et al. (2004). The range of the used NIR instrument is significantly narrower then the instruments usually used (with a range from 400 nm to 2500 nm), but to ensure the data differentiation between the samples that represent all stage in the wine production, for the further analysis is necessary to use some chemometric techniques. To predict expected concentration of ethanol, acidity, and glucose and fructose in white and red Prošek wines using NIR wavelengths: 1072, 1450 and 1696, but based on absorbance values although

Ozaki and co-workers (2007) have indicated 6 different wavelengths the restriction of the NIR instrument with the range of 904 – 1699 nm was the reason of reducing the number of NIR wavelengths on just 3. The linear model was used with the following form:

$$y_i = a_i \cdot AU_{i,1072} + b_i \cdot AU_{i,1450} + c_i \cdot AU_{i,1696} + d_i \quad \text{where } i = 1,..,4 \qquad [1]$$

i=1 ⇒ concentration of ethanol; *i=2* ⇒ concentration of acidity; *i=3* ⇒ concentration of glucose; *i=4* ⇒ concentration of fructose; *a,b,c d* ⇒ constants that differ regarding the *i*;
AU ⇒ absorbance unit for specific wavelengths given in the subscript.

In figures 2 and 3 is presented the agreement between the experimental data and values of the concentration of the equation 1. Following results indicated high correlation with the chosen wavelength what implies the possibility to predict the chosen concentrations of ethanol (*i=1*), acidity (*i=2*), (glucose (*i=3*), fructose (*i=4*).

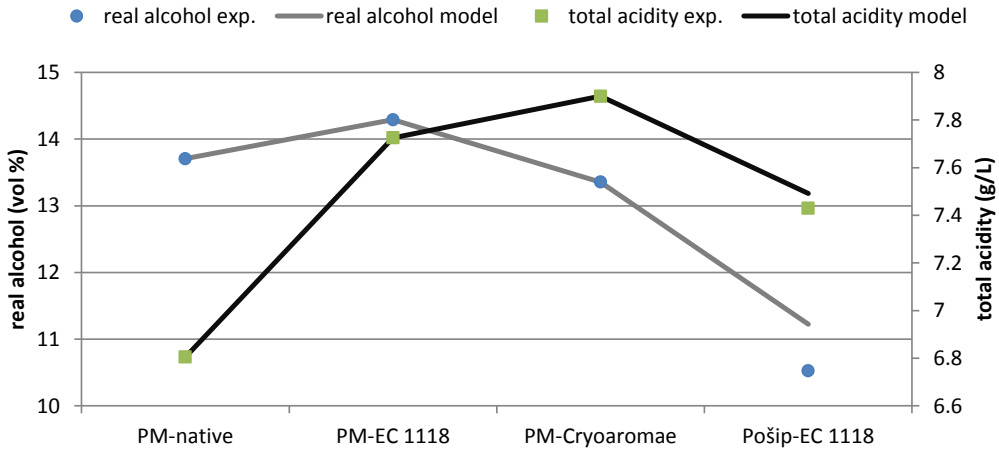

Figure 2. The agreement of experimental data and values gained with the model for real alcohol and total acidity

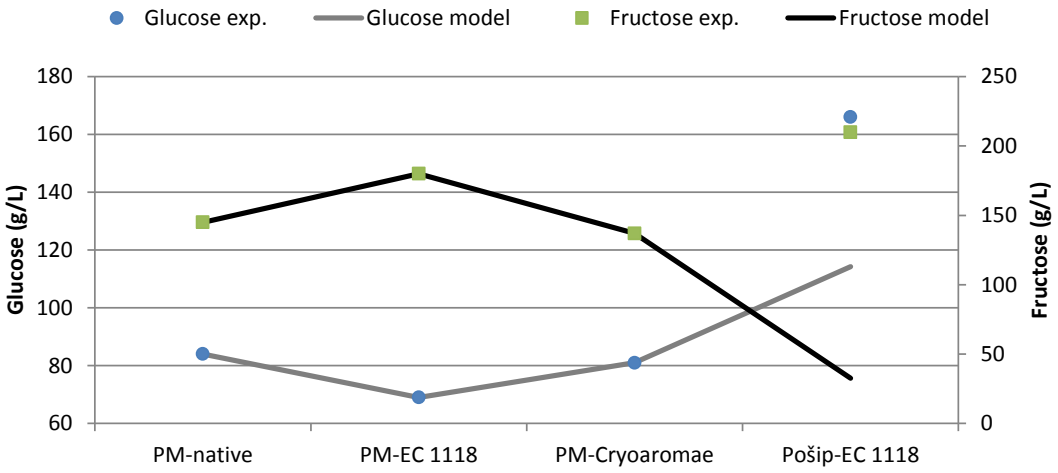

Figure 3. The agreement of experimental data and values gained with the model for glucose and fructose

In summary, this work annotated the advisability of use of NIR for on-line monitoring of product quality and selection (Alishahi et al., 2010; Nicolaï et al., 2007). It has also demonstrated the potential of NIR spectroscopy for prediction of chosen concentrations that are characteristic of quality of the wine Prošek what gives now the NIR spectroscopy also the

quantitative characterisation beside the qualitative characterisation of an observed product (Müller and Steinhart, 2007).

CONCLUSIONS

Near infrared spectroscopy showed promise as a rapid, non-destructive method in prediction of oenological parameters as concentration of ethanol, acidity, glucose and fructose for the desert wine Prošek. But the prediction of concentration of oenological characteristics using just 3 wavelengths (1072, 1450 and 1696) was more effective for red wine Prošek (produced prom cv. Plavac mali) than for the white wine Prošek (produced from cv. Pošip). This paper has presented that NIR spectrums and their specific wavelengths can be a useful tool for quality control. NIR can be used qualitatively to detect, to identify, and to qualify which grape was used in the wine production as well as the quantity of chosen oenological parameters of wine Prošek.

ACKNOWLEDGEMENTS

Presented results are derived from the project “Biotechnological Parameters of Premium-Quality Dalmatian Dessert Wine – Prošek”- (091-0910468-0452) and the project “Mathematical modelling, optimisation and process control in biotechnology” (058-1252086-0589) both supported by the Ministry of Science, Education and Sports, Republic of Croatia.

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ATTITUDES OF VLASINA HONEY PRODUCERS TOWARDS GEOGRAPHICAL INDICATIONS

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ABSTRACT: The Republic of Serbia is known as a producer of wide range honey varieties. The Vlasina plateau in the south part of Serbia covers the area of 12740.90 ha. According to the Regulation of the Government of the Republic of Serbia ("Official Gazette of RS", no. 30/06) Vlasina area is protected as a landscape of outstanding features and categorized as a natural resource of great importance for the Republic.
The diversity and specificity of biotopes Vlasina areas have caused a high diversity of flora, vegetation, fauna and ecosystems that are characterized by high degree of origins and authenticity of natural features. The area is habitat for a number of natural rarities, as well as natural phenomena. Beekeepers in the area mainly deal with stationed beekeeping in beekeeping directly at Vlasina plateau.
The aim of this paper was to find out whether producers of Vlasina honey are familiar with geographical indications (GIs) and to determine their perception and attitudes about these indications. The article presents the results of a research that shows that only a small part of producers are familiar with GIs. This group believes that GIs would have positive effect on honey competitiveness and especially on marketing, increasing sales prices and reduce a black market. The results suggest that there is a need of better information regarding GIs.
Key words: *GIs, Vlasina honey, marketing*

INTRODUCTION

Vlasina is the plateau in the Republic of Serbia with good natural conditions and moderate continental climate as well as richness of flora which provides excellent conditions for the bee-keeping and honey production. Honey is healthy product and one of the few products that can be consumed without any human processing (Ványi et al. 2011).
According to official data annual honey production in Serbia is approximately 4500 tonnes (Statistical Yearbook of the Republic of Serbia). This indicates that total value of honey production doesn`t has significant contribution to the Gross Domestic Product in Serbia but other benefits play more important role. At the present time bee-keeping is most commonly family business and one of the additional activities which can provide alternative income (Ostojić et al. 2011).
In order to improve marketing of their own products and achieve recognition on the market, domestic producers of agricultural products are increasingly interested for protection of geographical indication of products (Mesić et al. 2010).
Geographical indications as a marketing tool contribute to commercial value of products or services and increase the potential of selling with higher prices. With transmission of cultural tradition of the region and the specifics of the area in food production it can be create a unique product identity (Babcock and Clemens, 2004).
Primary objective of protection of geographical indications is to prevent the possibility of unfair competition. In the Republic of Serbia there are some products that are perceived by consumers as very specific for a certain region. In the Institute for Protection of intellectual property till now is protected a total of 58 products, of which 43 products are with domestic origin.

Encouraging the production of protected autochthonous products and their promotion could significantly contribute to the rural economy through the increase of income of farmers who are engaged in the production of these products. The procedure of protection takes time and financial resources and it is important that producers recognize the importance of protecting their products and the benefits arising from such protection.
The aim of this research is to examine attitudes of honey producers towards geographical indications.

MATERIAL AND METHODS

In this research a structured survey was conducted designed for bee-keepers in the area of Vlasina plateau in order to understand their attitudes towards GI. Sampling was carried out using a simple random sample through a survey, as an instrument of data collection. The research was conducted in 2011.
The survey included 86 honey producers who are bee-keepers on Vlasina plateau. Data were collected on age and gender, the size of production, the sales of honey, share of income from honey production in total income, if they are familiar with geographical indications and their opinion about possibility of conducting one for Vlasina honey. The data were analysed using descriptive statistics.

RESULTS AND DISCUSSION

This research included 88% men and 12% of women respondents. More than 60% of respondents aged over 50 years which indicates unfavourable age structure. Most of the respondents have finished high school (47.2%), but the number of interviewed with higher education is slightly lower (45.8%).
Total annual production and sale of honey is showed in table 1.

Table 1: Total annual production and sale of honey

	Mean	Mode
Produced (kg)	989	500
Sold (kg)	644	400
Price (RSD)	360	400

On average every bee-keeper produces about one tone of honey per year, of which approximately 70% is sell on the market and remaining part is home consumption. It is important to underline that most bee-keepers produces only 500 kg honey per year. These provide us information that producers on Vlasina are small scaled.
The share of income from honey in total income by the most survey producers is medium (51%). Only 22 % stated that honey production has large or very large share in their income, which leads to the conclusion that this group consists of commercial honey producers. About quarter of producers stated that they have very low (8%) or low (19%) income from honey production (Table 1). The relative importance of income provides useful information regarding attitudes towards GIs.

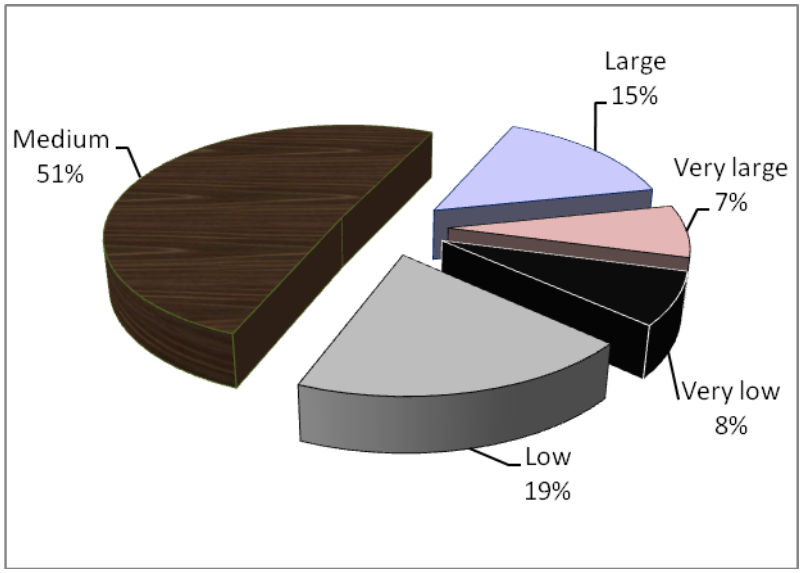

Figure 1. The share of income from honey in total income

Regarding possibility of protection of GI only 15% think that is very promising idea and 34% that is promising idea, and the remaining part of 51% has opinion that is not promising or bad idea (Table 2). This results show us that producers from Vlasina honey are divided into two equally groups, pro and contra GIs. This is probably results of the fact that process of geographical protection cause cost and producers are not sure of the benefit. In addition producers are not sure how protection of GIs would protect from unfair competition.

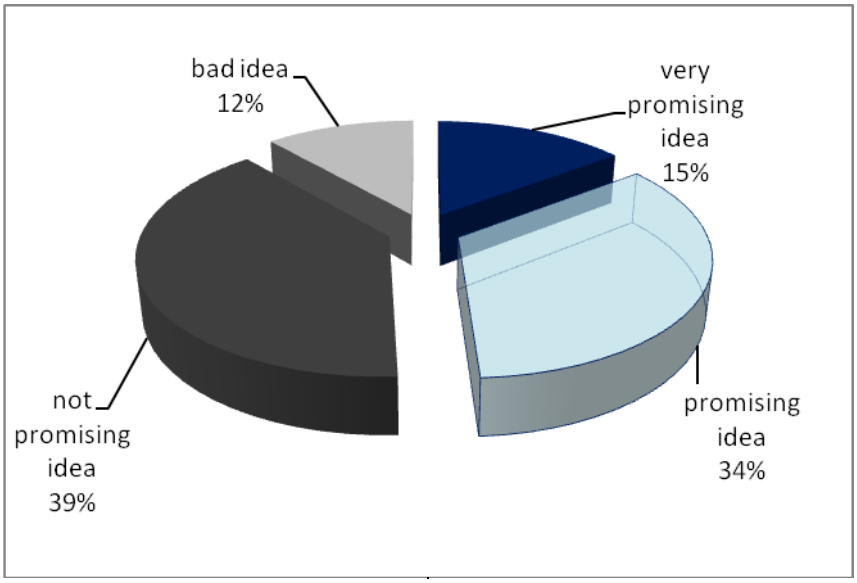

Figure 2. Attitudes towards possibility of geographical indications

CONCLUSIONS

The Vlasina plateau has high diversity of flora and fauna and specific ecosystem. The area is habitat for a number of natural species, as well as natural phenomena. The area is known by producing of high quality honey. Bee-keepers in the area mainly deal with stationed beekeeping in beekeeping directly at Vlasina plateau. As a rule bee-keepers are small in size and honey production is addition activity. Producers are aware of the importance of GI, but only a half of them think that protection of GI is a good idea. This is probably because of the relative high cost of protection and insecurity regarding further benefit of this activity. In this

case the incentive from institution could contribute to the development of positive attitudes towards GIs.

ACKNOWLEDGEMENTS

This paper is the result of the research financed by the Ministry of Education and Science of the Republic of Serbia.
Project number: III46001: "Development and implementation of new and traditional technologies in the production of competitive food products with added value for European and world markets - create wealth with Serbian wealth". The research period of this project is from 2011th to 2014th year.

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THE USE OF FOOD SAFETY OBJECTIVES AND RELATED CONCEPTS IN MICROBIOLOGICAL RISK ANALYSIS

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ABSTRACT: Foodborne illnesses - especially those caused by microbial hazards - have major public health consequences worldwide. In order to ensure continuous improvement in the health of population related to foodborne hazards, government is responsible for creating the Food Safety Policy. On international level, large efforts have been made to develop certain tools to link specific food safety requirements with its effect on public health. Risk analysis framework, laid down by the Codex Alimentarius during the past two decades, linked food safety and public health through risk assessment. Based on the formal approach to risk analysis, Appropriate Level of Protection, Food Safety Objective, Performance Objective, Performance Criterion and Microbiological Criterion are concepts evolved for purpose of risk management. However, the use of these relatively new concepts in Microbiological Risk Analysis is still quite limited and inconsistent.
Key words: *Microbiological Risk Analysis, Food Safety Objective*

INTRODUCTION

Foodborne illnesses are a real and formidable problem worldwide, causing great human suffering and significant economic losses. In industrialized countries, it is estimated that up to one third of the population are affected by foodborne diseases each year (Kaferstein *et* Abdussalam, 1999; Schlundt *et al*., 2004). Therefore, food safety is a fundamental public health concern and achieving a safe food supply poses major challenges for international and national food safety officials. An array of biological, chemical and physical hazards pose risks to human health and obstacles to international trade in foods. It is generally accepted that biological hazards - primarily microbiological, such as *Salmonella*, thermophilic *Campylobacter*, *Yersinia enterocolitica*, verotoxigenic *Escherichia coli* and *Listeria monocytogenes* - represent the highest foodborne risk for human health nowadays (Pointon *et al*., 2006; EFSA/ECDC, 2012). To protect and improve health of population, these risks need to be assessed, managed and communicated through the process of Microbiological Risk Analysis. In order to start this process, the government is responsible for creating the Food Safety Policy. To link specific food safety requirements with their effects on public health, certain tools have been developed during the last two decades (Figure 1). These tools are briefly described in this paper.

MICROBIOLOGICAL RISK ANALYSIS

Risk analysis, i.e. Microbiological Risk Analysis in the context of this paper, is a systematic, disciplined approach for making food safety decisions. It is a powerful tool for carrying out science-based analysis and for reaching sound, consistent solutions to food safety problems related to microbiological hazards. This process enables authorities to identify the various points of control along the food chain at which measures could be applied, to weigh up the costs and benefits of these different options and to determine the most effective one(s).
Microbiological Risk Analysis comprises three components: risk management, risk assessment and risk communication. Risk assessment is a scientifically based process consisting of the following steps: a) hazard identification; b) hazard characterization; c)

exposure assessment; and d) risk characterization. Risk management is the process of weighing policy alternatives in the light of the results of risk assessment and, if required, selecting and implementing appropriate control options (prevention, elimination, or reduction of hazards and/or minimization of risks), including regulatory measures. Risk communication is the interactive exchange of information and opinions concerning risk and risk management among risk assessors, risk managers, consumers and other interested parties (CAC, 1999).

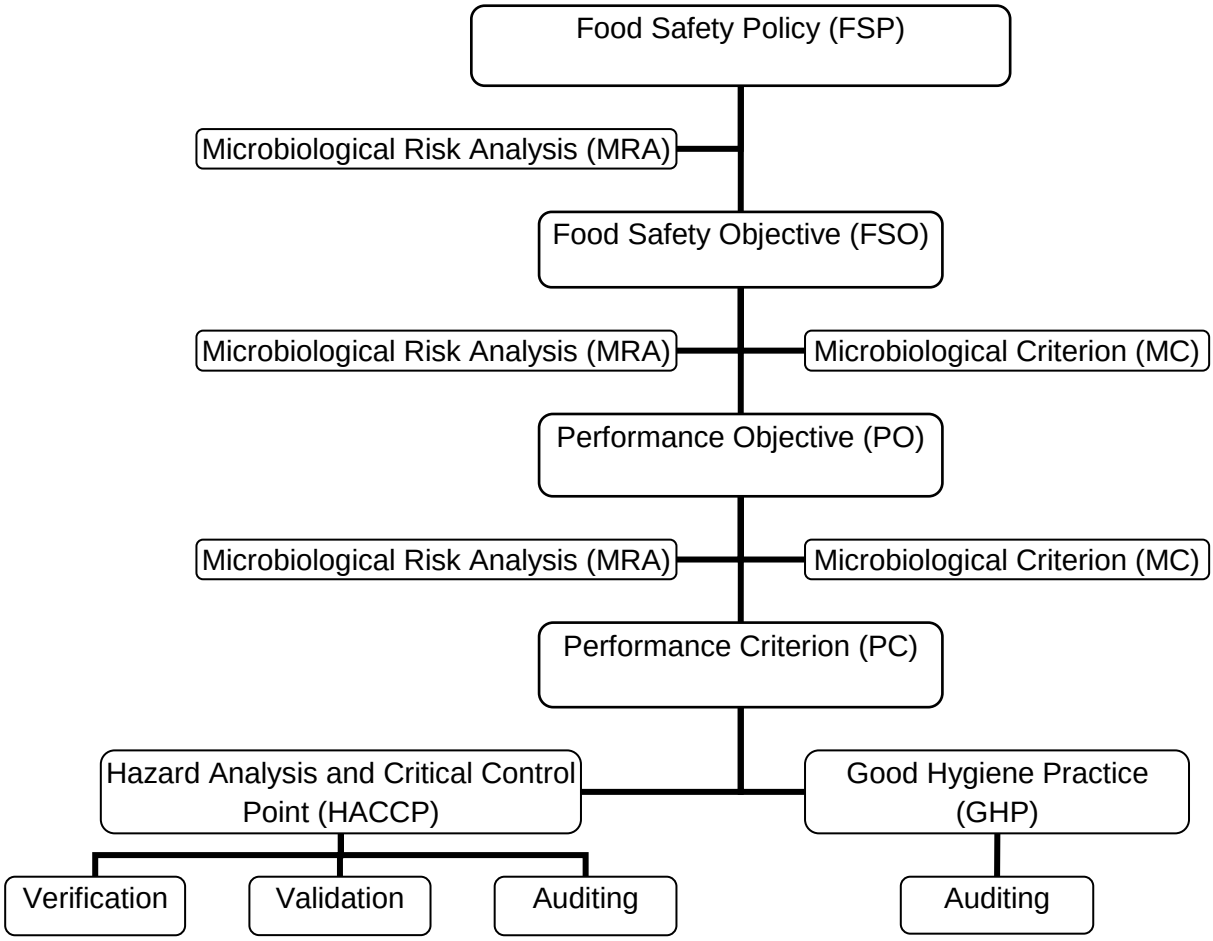

Figure 1. Food Safety Objective concept

APPROPRIATE LEVEL OF PROTECTION

Appropriate Level of Protection (ALOP) is defined by the World Trade Organization as the level of protection deemed appropriate by the member (country) establishing a sanitary or phytosanitary measure to protect human, animal and plant life or health within its territory (WTO, 1994). ALOP can be directly derived from risk assessment results and represents current public health status related to food safety, but can be changed during time (e.g. use of new technologies can change level of some food contaminant in the food chain). Instead of trying to eliminate all hazards from the food chain (i.e. final step of the food chain), ALOP represents the opinion that public health is improved by setting a goal and then determining the frequency and/or level of hazard in food that is compatible with that goal (Zwietering 2005).

Sometimes, ALOP has also been called "acceptable level of risk" (FAO/WHO, 2004); this term is similar to the expression "tolerable level of risk" preferred by the International Commission on Microbiological Specifications for Foods (ICMSF), because it recognizes that risks related to the consumption of food are seldom accepted, but at best tolerated. ALOP can be expressed at different levels. The most common level is total population of one

country, but sometimes it is better to express it on smaller, more specific population (e.g. if only small part of a population consumes certain type of food). ALOP can be general or specific; an example of general is incidence of some foodborne disease (e.g. salmonellosis) in population, and an example of specific is incidence of foodborne disease related to specific food (e.g. salmonellosis attributed to pork).
While ALOP represents current public health status related to food safety, "public health goals" are made to encourage further and future actions to improve public health by decreasing level of foodborne diseases. The concept of public health goals is recently introduced and still not formally defined by Codex Alimentarius. For that reason the difference between public health goals and ALOP is still not clear enough and sometimes these two terms overlap. Public health goals are usually set by the government or authorities of the country responsible for public health. An example of this concept is the target set by the UK Food Standards Agency in year 2001: the reduction of foodborne diseases by 20% by year 2006 (FSA, 2001), which was almost accomplished (Bell, 2006).

FOOD SAFETY OBJECTIVE

To be of use, the ALOP need to be translated into measurable objectives for the food industry. The concept of the Food Safety Objective (FSO) was developed to link the ALOP to food safety systems used in food manufacturing, and to link risk management with its effect on public health (Figure 2) (ICMSF, 2002; Gorris, 2005). Food Safety Objective is defined as the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the Appropriate Level of Protection (CAC, 2001), i.e. it is related to the point of the food chain when the hazard level can no longer change (ICMSF, 2002). This definition has been considered feasible for some ready-to-eat (RTE) food products with such intrinsic/extrinsic characteristics that restrain the growth of pathogens (Gorris, 2005; Walls *et* Buchanan, 2005; Zwietering, 2005), but not realistic for products that had undergone some treatment (e.g. cooking) at consumer level prior to consumption (Nauta *et* Havelaar, 2008). Also, the current definition relating to the moment of consumption has not been considered compatible with risk assessment methods and results and the value of an FSO derived from a Quantitative Microbiological Risk Assessment (QMRA) model has also been considered questionable (Havelaar *et al*., 2004).
Food Safety Objectives, defined as a part of the risk management process, should be used to govern Hazard Analysis and Critical Control Point (HACCP) system as an outcome definition (Schlundt 1999). Whenever possible, FSOs should be quantitatively expressed and subjected to verification. An example of an FSO is the level of *Listeria monocytogenes* in RTE foods that must not exceed 100 cfu/g at the time the food is consumed or the amount of staphylococcal enterotoxin in cheese that must not exceed 1μg/100g. However, this does not mean that FSOs must be verifiable through microbiological testing. For example, an FSO for low acid canned foods might be established in terms of the probability of a viable spore of *Clostridium botulinum* being present as being less than 0.000000000001 per can. It would be impossible to verify this by end-product testing, but it would be verifiable by measurement of time/temperature protocols that are based on a Performance Criterion (FAO/WHO, 2006).

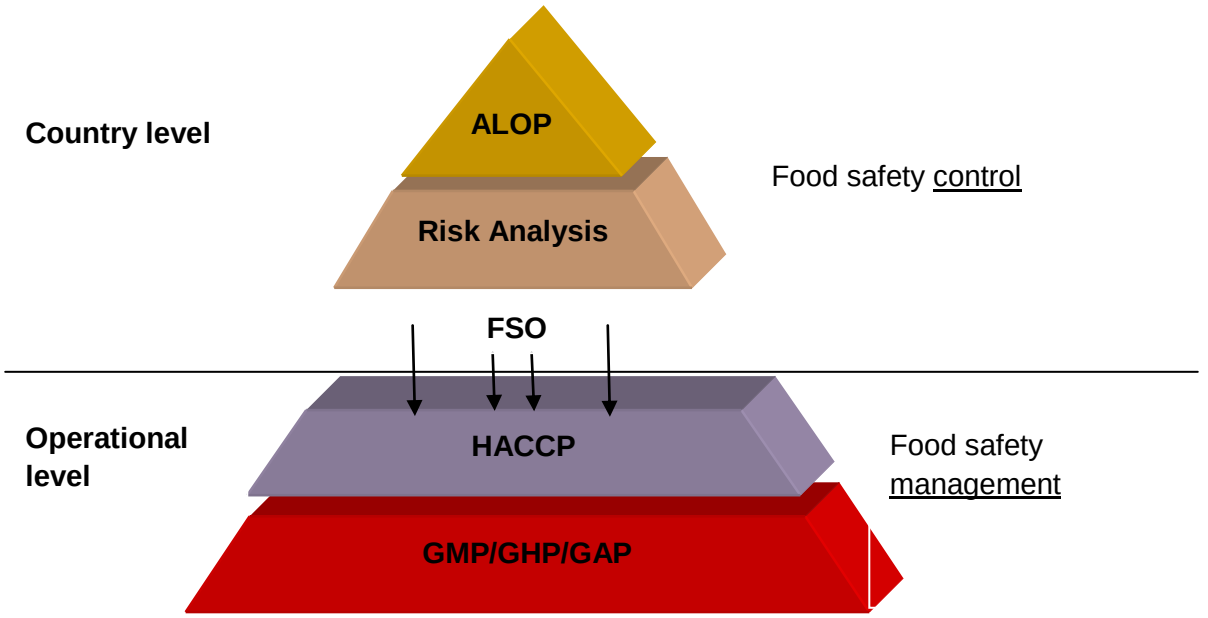

Figure 2. FSO as a link between food safety control on a country level and food safety management on an operational level (Gorris, 2005)

PERFORMANCE OBJECTIVE

As well as for an FSO, the original purpose of a Performance Objective (PO) was to translate an ALOP into levels of hazards in food chain that can be communicated and managed by the food industry. Performance Objective is defined as the maximum frequency and/or concentration of a hazard in a food at a specified step in the food chain before the time of consumption that provides or contributes to an FSO or ALOP, as applicable (WTO, 1994). In contrast to an FSO, a PO can be utilised at points of the food chain where control and verification are possible (FAO/WHO, 2006), and FSO is the value that should lead to the development of PO values earlier in the food chain (Gorris 2005). Also, in QMRA, FSO can be considered as the last PO - before application of the dose response model (Nauta *et* Havelaar, 2008). A PO may be established by the government or an individual food production company may establish its own POs.
Quantitative relationship between an FSO and a PO is different in different situations. When it comes to food that is heat-treated immediately before the consumption, an FSO is more stringent than a PO. For example, the FSO for *Salmonella* in heat treated chicken meat can be "absence in serving at the point of consumption". Since it is likely that a certain number of broiler chickens will be infected with this pathogen and that certain proportion of the broiler carcasses will be contaminated consequentially, it is possible to set up a following PO: maximum of 15% carcasses contaminated with *Salmonella* at the end of chilling process. In subsequent stages of the food chain, with appropriate heat treatment and Good Hygiene Practice (GHP), a given FSO can be achieved. On the other hand, regarding ready-to-eat food, an FSO and a PO may represent the same value or even a PO can be stricter than an FSO - for example, in the case of RTE foods with a relatively long prescribed shelf life, which support growth of *Listeria monocytogenes*.

PERFORMANCE CRITERION

Performance Criterion (PC) has been defined as the effect in frequency and/or concentration of a hazard in a food that must be achieved by the application of one or more control measures to provide or contribute to a PO or to an FSO (CAC, 2008). An example of a PC is a 6D reduction of *Salmonella* spp. when cooking ground beef (Cole, 2004). A Performance

Criterion specifying the frequency and/or concentration of a pathogen is identical to the "acceptable level" to be achieved at a Critical Control Point (CCP) in the context of HACCP.
Process Criterion (PrC) is defined as the physical process control parameter (e.g. time, temperature) at a specified step that can be applied to achieve a Performance Objective or Performance Criterion (CAC, 2005). For example, the control parameters for milk pasteurization in the USA are 71.7°C for 15 sec. This combination of temperature and time will assure at least 6 log reduction of *Listeria monocytogenes* and destruction of *Coxiella burnetii*, as well as other non-spore forming pathogens that are known to occur in raw milk (ICMSF, 1996; Cole, 2004). Process criteria are identical to critical limits on CCPs in a HACCP plans (CAC, 1997b). Performance and process criteria belong to the risk management measures that are to be applied on the operational level (e.g. food producing company) in order to carry out functional risk-based food safety management.

MICROBIOLOGICAL CRITERION

Microbiological Criterion (MC) is a criterion defining the acceptability of a product or a food lot, based on the absence or presence, or number of microorganisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot. In contrast to FSOs and POs which only represent limits, an MC consists of several aspects: a) the microorganisms of concern have to be stated, b) a qualitative or quantitative analytical method validated and chosen to give a sufficiently reliable estimate, c) critical limits based on data appropriate to the food, and d) a sampling plan including the sampling procedure and decision criteria for a lot (CAC, 1997a). The authorities can use these criteria to define and check the compliance of food production with the microbiological requirements, and food business operators can use them for verification and validation purposes. An MC may be set by a competent authority or industry and then implemented as a food safety measure using GHP or HACCP approaches (FAO/WHO, 2002). MCs are not derived from risk analysis but rather from management systems controlling foodborne hazards during food processing, determining the acceptability of specific production lots of food (ICMSF 2002).
In the EU legislation (EC, 2005; EC, 2007) an MC concerning the acceptability of food products on the market is termed Food Safety Criterion (FSC), while Process Hygiene Criterion (PHC) provide guidance on and is indicator of the acceptable functioning of HACCP-based food safety system. An example of an FSC is *Salmonella* absence in 25 grams of each of 5 samples of minced meat and meat preparations made from poultry meat intended to be eaten cooked, during their shelf-life of these products placed on the market. An example of a PHC is a presence of *Salmonella* in a maximum of seven of 50 tested neck skin samples derived from 10 consecutive sampling sessions of poultry carcasses after chilling.

CONCLUSION

Food Safety Objective concept is a powerful tool in risk management process which assists governments in achieving health goals throughout the food chain. It requires an integrated approach of risk assessment, risk management and risk communication. This concept is not only useful for protection/improvement of public health with respect to foodborne illnesses - it is a necessary part of modern, longitudinal and integrated food safety system. However, the use of this concept is still limited and inconsistent.

ACKNOWLEDGEMENT

This paper is a result of the research within the project TR31034, financed by the Serbian Ministry of Education and Science.

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RAPESEED MEAL AS A BY-PRODUCT FROM BIODIESEL PRODUCTION AS A FEED INGREDIENT

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ABSTRACT: Rapeseed/canola (*Brassica napus L.)* is one of the most important edible oilseed crops in the world, as well as a major potential of bio-diesel production in Europe. High oil content is one of the most important characteristics of this crop. Rapeseed meal, which is a by-product of oil extraction, is a highly rich raw material and contains up to 50% protein on dry basis. Plant proteins are largely used in the food and feed industry, and rapeseed proteins are regarded as potential ingredients that may be used as a food and feed additives.
In this paper the quality of rapeseed meal obtained during the biodiesel production was assessed and its use as a nutrient in domestic animals was discussed.
Key words: *rapeseed, by-products, biodisel*

INTRODUCTION

Rapeseed/canola (*Brassica napus L.)* is one of the most important edible oilseed crops in the world, as well as a major potential of biodiesel production in Europe. High oil content is one of the most important characteristics of this crop. Canola seeds contain approximately 38-45% oil and 17-26% proteins. Oil belongs to a group of semi-drying oil with iodine value 95-120. It is used in nutrition and for technical purposes. Technical oil is used in the industry of soap, paint, textile, leather, in printing and as an additive to lubricants. After extraction of oil remains the meal/cake which is used as animal feed. Rapeseed can be used as animal feed in green condition. In the green mass of oilseed rape there is more digestible protein than in corn, sunflower, winter wheat and rye, oats, sudangrass, but also more than in alfalfa. In relation to the mass of green alfalfa, rapeseed contains nearly two times less crude fiber (Marinkovic et al., 2009).
Development of mobile systems has been based on the use of fossile fuel to a great extent. The changes in fuel prices imposed needs for fast discovery of an alternative energy sources. Biodiesel is an adequate solution because it is compatible with fossile fuels, based on restorable raw material and ecologically more acceptable than fossile fuels. The techology of biodiesel production consists of processes of oil expression and reesterification. Meals and cakes – the sources of protein in feed - are obtained as important second products in biodiesel production. Interest with rapeseed press cake as the by-product of the rapeseed oil industry has been increasing along with its potential of being a renewable raw material.
Plant proteins are largely used in the food and feed industry, and rapeseed/canola proteins are regarded as potential ingredients that may be used as food and feed additives. Canola meal, which is a product of canola oil extraction, is a highly rich raw material. The major protein constituents of canola meal are napin and cruciferin, which are storage proteins, and oleosin, which is a structural protein associated with the oil fraction (Uppstroom, 1995). The amino acid composition of canola meal is well balanced and can be used for human nutrition (Aider and Barbana, 2011). Some properties of canola proteins were comparable to those of casein and better than those of other plant proteins, such as soybean, pea and wheat (Ghodsvali et al., 2005). Soy contains more lysine than the rapeseed, and rapeseed seeds contain more sulfur amino acides (Aherne and Kennelly, 1985). The use of canola protein is

limited by the presence of some undesirable compounds, such as glucosinolates, sinapine, phytases and phenols, which are responsible for the toxic, antinutritional and undesirable capacity of canola proteins (Aider and Barbana, 2011). Also, canola meal contains approximately 20% (w/w) carbohydrates, including soluble sugars (Lacki and Duvnjak, 1998). Fibres including cellulose, pentosans and lignin from cell walls, are mainly present in the hulls of canola. High fibre contents in animal feeds decrease its digestibility, thus decreasing the value of the feed (Mailer et al., 2008). However, sinapine remains at levels sufficiently high to cause problems, particularly in poultry feed. Its presence in the diet of brown egg laying hens at levels exceeding 1 g/kg leads to a fishy odour in the eggs (Butler et al.,1982).
Some authors suggest that the palatability of rapeseed products is a limiting factor for their use in complete feed mixutres (Bell et al., 1988). Bitter taste is the result of the presence sinapine in concentration 1 to 1.5% (Straková et al., 2008). Sinapine is a choline ester of sinapic acid which is important in plants for the biosynthesis of lignin and flavonoids (Mailer et al., 2008). Canola breeding programs have succesfully reduced glucosinolates content to trace amounts. New varieties of rapeseed are labeled "00" and for them the name "Canola" is introduced. “Canola” varieties contain less than 1% erucic acid (maximum 2%) and lesser than 20 μmol g^{-1} glucozinolates (maximum 30 μmol g^{-1}).
One of the most important factors determining the composition of oil cakes as feedstuff is the processing method. As for most other oilseeds, pretreatment includes: crushing, cooking and, in rare cases, dehulling. Dehulling of rapeseed aims at the removal of the major part of the fibre and pigments, which otherwise would lower the feeding value of the meal/cake (Ohlson, 1992). As by-product of biodiesel cake is processed in one of the three ways: expression, extraction and expression-extraction combination. The most used proceedings for fat acids methyl ester, i.e. biodiesel synthesis, is the process of transesterification (cold discontinuous and continuous proceedings) (Furman et al., 2005).
The gradual increase in the world’s animal production implicates the need for ingredients to provide protein in diets. Canola meal, which is the product of canola oil extraction, is a highly rich raw materia, and as a second product in biodiesel production is a good energy source. Taking into consideration EU directives that it is necessary to substitute fossil diesel with 0.75% of biofuel per year, the possibility to use the by-products of this industry calls for attention. In this paper the quality of rapeseed cake obtained during the biodiesel production was assesed and its use in animal nutrition was discussed.

MATERIAL AND METHODS

Materials

Testing the quality of oil cake, obtained during the biodiesel production for animal nutrition, was carried out on 11 samples: 4 samples of rapeseed variety, type “Banaćanka”, 4 samples of rapeseed, type “Kata”, and 2 samples of rapeseed, type “Slavica”. Baseline characteristics of the investigated canola seeds are given in Table 1.

Table 1. Characteristics of the tested canola seeds

	Variety		
Property	„Banaćanka“	„Kata“	„Slavica“
Oil content (%)	46	46	44-51
Erucic acid content (%)	< 1.0	< 1.0	< 1.0
Glucozinolate content (mmol/g seed)	< 20	< 20	< 20
1000 grain weight (g)	4.2	4.0	4.3

Processing method

Drainage of non-shelled seeds was performed on a continual press "EKS BIO", the capacity 50 kg/h. The press consists of two parts: a screw conveyor and a basket. The role of the screw conveyor is to ensure the intake of seeds and to push them into the basket. Thus it increases the pressure and enables that the squeezed oil drains through the holes in the basket. At the end of the screw conveyor the cake comes out through a ring opening. The

drainage of oil was carried out at different conveyor speeds and by using different diameters between the end of the screw conveyor and the ring opening. The variations in these parameters were carried out in order to obtain the optimal ratio of the squeezed oil and to achieve optimal effect of the presses. After squeezing the obtained second product (cake) was analyzed as a possible feed for animal nutrition.

Chemical anlaysis

Determination of fat, crude fiber, ash and moisture in the samples was performed by standard methods, while the protein was analyzed by measuring total nitrogen by total combustion (according to Dumas), as the standard method (AOAC 990.03) on the instrument „Elementar, Rapid N cube“. The contents of nutrients was expressed in percentages. The results were statistically analyzed. Depending on the results a conclusion will be given for the following steps in research of the byproducts of biodiesel production.

RESULTS AND DISCUSSION

The values of basic nutrients are presented in Table 2. The obtained results suggest that by-products of biodisel production can be used as feedstuffs for domestic animals.

Table 2. Chemical composition and % of expressed oil of investigated rapeseed cakes

Hybrid type	Sample No	% of expressed oil	*Investigated parameter* (%)				
			Crude protein	*Crude fiber*	*Crude fat*	*Crude ash*	*Moisture*
Banaćanka	1	20	23.17	11.69	30.66	5.23	6.12
	2	30	25.26	12.62	21.56	5.90	6.83
	3	35	26.99	13.69	15.92	6.39	6.62
	4	25	25.69	13.80	21.70	5.77	6.40
$\bar{X} \pm Sd$		n.a.	25.28±1.59	12.95±0.99	n.a.	5.82±0.48	6.49±0.30
Kata	5	14	27.32	10.53	29.11	4.42	6.28
	6	35	33.79	12.35	11.77	5.46	7.00
	7	20	28.59	10.52	24.56	4.60	6.76
	8	25	31.21	11.62	18.79	4.89	6.52
	9	30	31.55	11.36	18.38	4.99	6.68
$\bar{X} \pm Sd$		n.a.	30.49±2.56	11.28±0.78	n.a.	4.87±0.40	6.65±0.27
Slavica	10	27	31.17	12.55	14.42	5.72	6.04
	11	23	28.71	9.16	19.34	5.39	6.56
$\bar{X} \pm Sd$		n.a.	29.94±1.74	10.86±2.40	n.a.	5.56±0.23	6.30±0.37

N.A. not statistically analyzed

The levels of investigated components in rapeseed cakes varied as follows: crude proteins - 23.17 - 33.79%, crude fibre 10.52 – 13.80, moisture 6.04 – 7.00, crude ash 4.42 – 5.90 and crude fat 11.77 – 30.66%. The products obtained by applied procedure have high oil content. The total oil content (expressed+crude fat in meal) in seed of canola for the examined varieties ranged as follows: „Banaćanka“ - 46.70 – 51.56%; „Kata“ - 41.42 – 42.34% and „Slavica“ - 37.42 – 46.34%. The obtained results are in accordance with other relevant investigations (Marinković et al., 2009; Straková et al., 2008; Mailer et al., 2008; Uppstrom, 1995 ; Karaosmanoğlu et al., 1996;).

Oils are important dietary ingredients in animal production owing to their high energy value. Futhermore, the fatty acid (FA) pattern of the dietary lipids is reflected in the fatty acid profile of animal products (Jørgensen et al., 2000). Public health concerns are driving research into modifyng the fatty acid profiles of milk and meat, particularly toward less saturated medium chain and more long-chain polyunsaturated fatty acids. The main source of unsaturated lipids are oilseed lipids, among which is the rapeseed used in animal nutrition. Rapeseed oil has moderate levels of 18:2 (n-6) FA and 18:3 (n-3) FA, in ratio of 2:1, and an abundance of 18:1 (n-9) FA. The ratio of 18:2 (n-6)/18:3 (n-3) in rapessed oil makes it of benefit to human and animal health (Gordon Bell et al., 2001).

Crude protein and crude fibre content variance are related to the applied procedure of oil extraction from rapeseed. Comparing to sunflower cake (Table 3) produced in a similar way, rapeseed cake in average has high protein content and low crude fibre content, what makes it a desirable raw material for feed production.

Table 3. Chemical composition and % of squeezed oil of sunflower cakes (Živkov-Baloš et al., 2011)

Hybrid type	*Investigated parameter* (%) $\bar{X} \pm Sd$			
	Crude protein	*Crude fiber*	*Crude ash*	*Moisture*
"Nicholas"	28.13±2.78	15.74±1.12	5.89±0.55	6.57±0.44
"Oliva"	24.46±4.49	17.71±3.02	5.11±0.87	6.44±0.75
"Somborac"	21.88±2.17	18.07±2.26	4.88±0.67	6.14±0.49

CONCLUSION

Considering the fact that biodiesel is made of restorable raw material (plant oils), and that there are some insignificant differences in the energy potential of the fuel, it is quite obvious that biodiesel is the right solution for future. Biodiesel has great advantages in ecological sense. Therefore, in the forthcoming years the exploitation capacity of its by-products should be evaluated as relatively inexpensive protein and energy sources for animal diets. The following factors need to be considered to ensure maximum benefit from rapeseed:

- the efficacy of oil production procces needs to be enhanced;
- pelleting (temperature) helps to increase nutrient utilization;
- enzyme supplement has a positive effect on the nutrient utilization;
- efforts by plant breeding programs focused on rapeseed with improved sinapine, glucosinolates and fibre.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 031071 "Research on pharmacological characteristics of antimicrobial agents, introduction of new technological solutions and alternative prophylactic methods with the purpose to improve control of infectious animal disease" financed by the Ministry of Education and Science, the Republic of Serbia.

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THE VARIATIONS IN QUALITY OF MAIZE BIOMASS IN DIFFERENT ENVIRONMENTS

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ABSTRACT: Successful maize cropping in rein-fed conditions depends mainly on meteorological factors and in lesser extent on substrate (soil). The trial with 5 maize hybrids was set up during period of 2005-2010 on alluvium and hydromorphous black soil with aim to examine productive traits and silage quality from the energetic point of view. Biomass yield, stover and cob mass were measured, as well as total energy of biomass, as calculated parameter. Nutritive units, total energy and metabolic energy were determined from the silage.
Positive correlation was observed between yield parameters and precipitation amount. In most of seasons, biomass yield, stover and cob mass, total energy of biomass and silage were higher on hydromorphous black soil, compared to alluvium, while the nutritive units and metabolic energy were lower on the same soil during unfavorable seasons. Achieved results indicated that unfavorable meteorological factors could be partly reduced by cropping on heavier soils, such hydromorphous black soil is. In contrast to yield parameters, energy status of produced maize and silage biomass are in lesser extent dependable on meteorological conditions and soil type, and what is more important biomass total energy increases with unevenness of growing conditions. Such situation gives advantage to lower yield crop as a source for energy production (bio-fuels), while high yielding crop have better potential for conversion as a feedstuff.
Key words: *maize biomass, silage, energy status*

INTRODUCTION

Rain-fed cropping is still the most abundant maize cropping practice in many regions. From that point of view, meteorological conditions and soil condition has important position in biomass yielding. Studies of Iqbal et al. (2003) showed that cropping on clayey soil could increase fresh plant mass by better water using efficiency. On the other hand, successful cropping on light soils includes higher fertilization rates and irrigation (Sileshi et al., 2010).
Other than precipitation sums, which mainly affects maize biomass production, temperature factor has large role, too. Growth and development of whole organisms show a temperature response, because of the integrated effects of temperature on the physiological processes involved. Understanding maize growth in response to temperature has led to the concept of “growing degree days” (GDD) (Bonhomme et al. 1994). The GDD approach provides a unit for maize growth estimation in response to variable environmental conditions.
Maize growth and yielding includes capturing of the solar energy, which is 2 to 8 times higher under intensive modern management systems than in hand or animal powered systems (Pimentel, 1984). Produced biomass is main source of feed production and in recent time for energy production. Currently, the dominant source of biomass based liquid transportation fuels is ethanol from maize (Field et al., 2008). Increased production of biomass for energy has the potential to partly replace use of fossil fuels, but it potentially endangers conservation areas, water resources and decreases food security. On the other hand, energy production from the removed agro-residues could give sustainable solution, since the average energy output for food was always greater than that for fuel (Gelfand et al., 2010). The climate, geography and management of land are all factors in the calculation of the amount of

biomass able to be removed sustainably. Sites with steep slope, dry climates, and/or highly erodible soils produce much less and in some cases no removable organic matter. The low nutrients content of corn cobs reduce the amount of nutrients removed with cobs at harvest which later must be replaced to maintain optimal soil nutrient levels (Zych, 2008).
The aim of the study was to compare the influence of meteorological conditions and two soil types during six experimental years on maize yield components, as well as energy status of produced biomass and silage.

MATERIAL AND METHODS

The trial was set up in the PKB Corporation in Padinska Skela (44° 59' 52'' N, 20° 22' 18'' E, 67–69 m altitude) with five maize hybrids, Staniša, Dukat, Srećko, Rubin and Dijamant (FAO 300-600). The crops were established on alluvium (as a model of sandy, light soil) and hydromorphous black soil (as a model of clayey, heavy soil) under rain-fed conditions. The experiment was set up in a random block system on a total area of 216 ha in three replications with an elementary plot of 6 ha. The sowing was performed during April and first decade of May, depending on the meteorological conditions. The plants were cultivated at densities of 68,000-74,000 plants ha^{-1}. The maize harvesting for silage preparation was performed when the dry matter was 34–36%, depending on the meteorological conditions, i.e., during August-September. The cob and stover mass, as a yield parameters of 30 maize plants per replication were determined just before crop harvesting.
The harvested material was transported to pit silos where it was inoculated with Sil-ALL (Alltech, UK) at 1 g t^{-1} plant material and compressed properly. Again, the pit was covered with a plastic sheet and compressed with straw bales (weight 250 kg) for 75 days, when silage samples were collected (three samples per silo pit, at middle and the two ends).
The nutritive units (NU), based on silage composition, analyzed by "Standard accredited methods" (Official Gazette – Službeni list SFRJ 1987; AOAC 1990) were determined as Scandinavian feed unit (SFU), based on methods of Obračević (1990) and van der Honing and Alderman (1988), also the total (TE) and metabolic energies (ME) of silage were determined by method of Obračević (1990). Total energy (TE) of maize biomass was calculated based on thermodynamic data of biomass conversion, proposed by Domalski et al., (1986).
The growth degree days (GDD) were calculated by the formula proposed by Cross and Zuber (1972):

$$GDD = (T_{max} + T_{min})/2 - T_{base}$$

where T_{max} is the maximum daily temperature and was set equal to 30 °C when the temperatures exceeded this level, T_{min} is minimum daily temperature and was set equal to 10 °C when the temperature fall below this value, and T_{base} was taken as 10 °C for maize (Cross and Zuber 1972).
The experimental data of biomass yield, stover and cob mass and nutritive units were statistically processed by analysis of the variance (ANOVA) by the LSD-test (5%), while the GDD, total energy of silage and maize biomass, as well as metabolic energy of silage were presented with standard deviation (SD). Correlation between GDD, sum of precipitation and biomass yield, stover mass, cob mass, nutritive units TE of biomass and silage and ME were presented with significance level P = 0.05.

Meteorological conditions

Meteorological conditions during present period of 2005-2010 emphasized 2007 and 2008 as dry years, with lowest precipitation sum (Figure 1), almost double in relation to other years. Additional impact in 2007 was obtained in highest GDD, particularly caused by high temperatures present during vegetation. From that point of view 2007 was characterized as a unfavorable season.

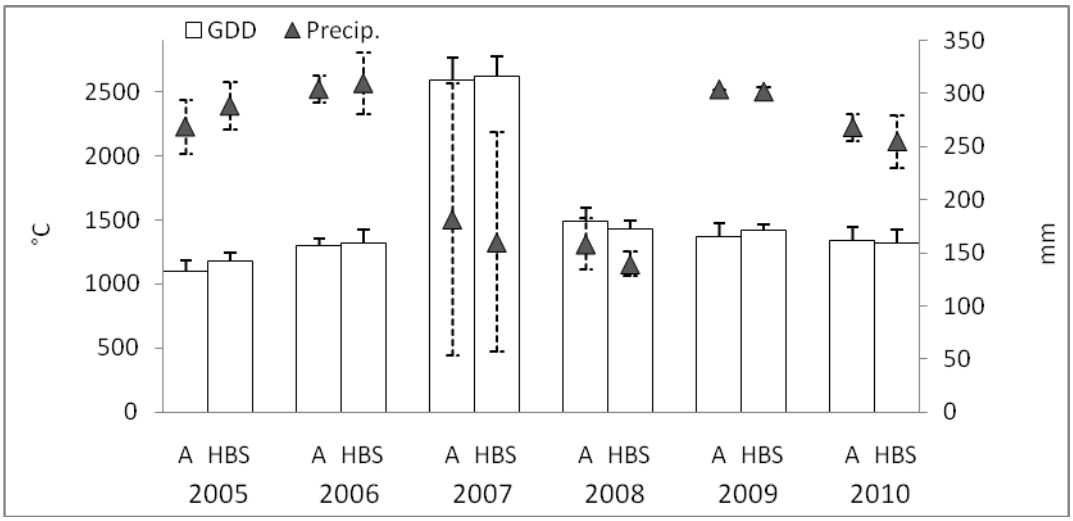

Figure 1. GDD-s and precipitation sum during vegetation period of 2005-2010

RESULTS AND DISCUSSION

The results of biomass yield, stover and cob biomass and nutritive units of produced silage (table 1) had higher, but insignificant average values on hydromorphous black soil. On the other hand, seasonal influences, present in alterations of meteorological factors (Figure 1) emphasized 2005, 2009 and 2010 as years with significantly higher values of produced maize biomass, as well as stover and cob biomass (table 1).

Table 1. Biomass yield, stover and cob mass and nutritive units of silage produced on alluvium (A) and hydromorphous black soil (HBS) during period 2005-2010

	Biomass yield			Stover mass			Cob mass			Nutr. units		
Year	A	HBS	Aver.	A	HBS	Aver.	A	HBS	Aver.	A	HBS	Aver.
2005	42.1	45.4	43.8	2070	2596	2333	389.9	392.8	391.4	0.33	0.36	0.35
2006	32.3	35.6	34.0	1221	1337	1279	326.4	367.1	346.8	0.37	0.36	0.36
2007	29.4	34.5	31.9	1257	1328	1292	294.4	362.9	328.6	0.37	0.33	0.35
2008	31.6	34.7	33.2	979	1084	1031	337.2	370.0	353.6	0.40	0.39	0.39
2009	42.0	44.6	43.3	1271	1458	1364	469.9	493.4	481.7	0.38	0.43	0.40
2010	42.9	42.9	42.9	1727	1881	1804	418.6	404.2	411.4	0.33	0.37	0.35
Aver	36.7	39.6		1421	1614		372.7	398.4		0.36	0.37	
LSD	Soil	Year	Int.	Soil	Year	Int.	Soil	Year	Int.	Soil	Year	Int.
0.05*	6.3	3.7	3.5	562	363	357	65.6	44.1	41.5	0.05	0.43	0.04

**Least significant difference, P = 0.05 (n = 3)*

Positive influence of hydromorphous black soil on a production of maize biomass was particularly underlined during 2005 on stover production and during 2009 and 2010 on cob production. Seasonal influences also had the greater impact on alterations of nutritive units in silage, opposite to soil influence, underlining 2008 and 2009 as seasons with higher nutritive units, in significant.

Energy status of produced maize biomass and silage is giving advantage to silage, according to silage/biomass TE ratio, which is varying from 1.68 (obtained on alluvium in 2007) to 1.98 (obtained on hydromorphous black soil in 2005, Figure 2). Fluctuations in energy of produced maize biomass and silage were minor between examined soil types (< 1% in average), indicating slightly higher values of silage TE and ME on hydromorphous black soil, as well as the lover values of biomass TE (6%, in average) on the same soil, compared to alluvium. Main influence, which induced increase in energy of produced biomass and silage, came from seasonal influence, with highest values of silage TE in 2008 and silage ME in 2005 and 2007, on average, while the highest values of biomass TE was obtained in 2007 and 2010, in average. The higher values of ME and TE of silage and biomass were obtained in years with

relative extreme conditions (Figure 1): high GDD (2007) and low sum of precipitation (2007 and 2008), meaning that favorable meteorological conditions for biomass yielding don't support its quality on energy basis. According to results of Haigh (1995), the highest ME values were obtained during dry and warm seasons, while the lowest values were noticed during wet and cool seasons. Moreover, no correlation was obtained between biomass yield and ME (Millner et al., 2005), indicating that factors which affect yield and its components doesn't affect energy of produced biomass, i.e. silage.

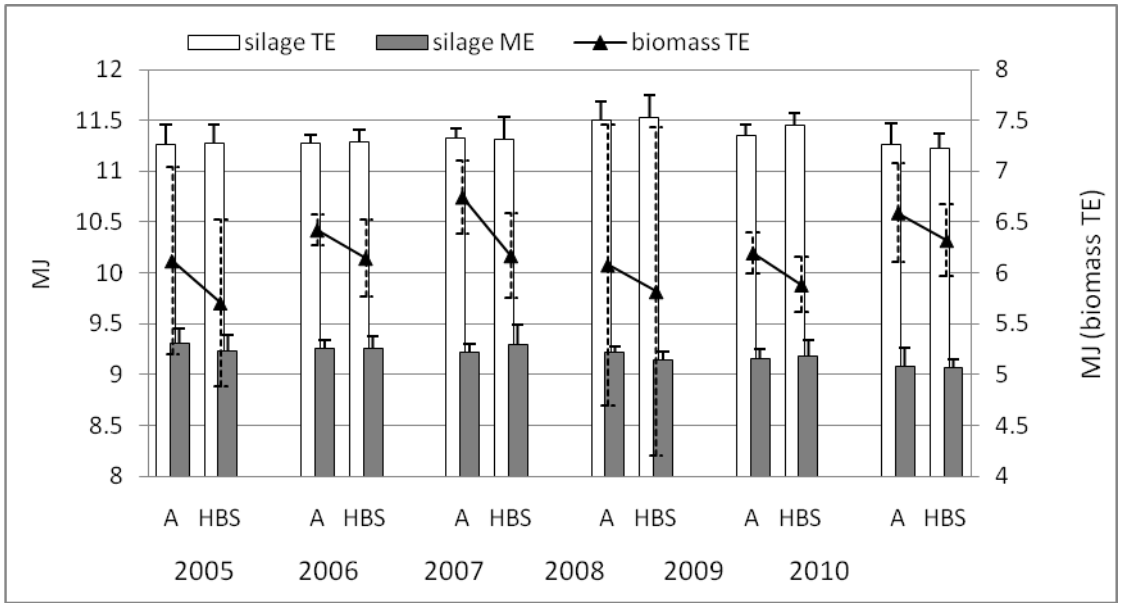

Figure 2. Total energy (TE) of silage and maize biomass and metabolic energy (ME) of silage produced on alluvium (A) and hydromorphous black soil (HBS) during period 2005-2010*; Results are given as mean ± standard deviation (n = 3)*

The variations of meteorological conditions, present in precipitation sum and GDD indicated significantly high impact of both factors on biomass yield (table 2): GDD expressed negative influence, while precipitation sum expressed positive influence. The significant negative correlation of GDD and yield parameters (biomass, stover and cob mass) signifies that the extended vegetation period or high temperatures could induce biomass losses (Herrmann et al. 2005). Precipitations affected stover and cob biomass in higher extent, while GDD was more important for alterations of cob mass. Obtained results confirm model purposed by Wolf and van Diepen (1994), which provided higher biomass yields for hybrids of shorter vegetation and northern parts of Europe (lower GDD-s) including limits in water supply. Opposite to negative correlation between GDD and yield parameters, positive correlation between GDD and biomass TE was observed, similarly to results of Haigh (1995). On the other hand high precipitation induced decrease of silage TE.

Table 2. Correlation coefficients between GDD, precipitation sum and biomass yield, stover mass, cob biomass, nutritive units total energy of silage and biomass and metabolic energy

	Biomass yield	Stover mass	Cob mass	Nutr. Units	silage TE	silage ME	biomass TE
GDD	-0.57*	-0.38*	-0.44*	-0.15	0.07	0.27	0.41*
Precip.	0.60*	0.43*	0.52*	0.00	-0.53*	0.02	-0.01

**Least significant difference, P = 0.05*

CONCLUSIONS

Concluding on based achieved results, meteorological factors presumably affect maize yield components and biomass yielding. Unfavourable climatic changes, present in increased temperature and lower precipitation level could be partly reduced by growing on heavier, clayey soils, such hydromorphous black soil is. In contrast to yield parameters, energy status of produced maize and silage biomass is in lesser extent dependable on meteorological conditions and soil type and that is more important biomass TE is increases with unevenness of growing conditions. Such situation gives advantage to lower yield crop as a source for energy production (bio-fuels), while high yielding crop has better potential for conversion as a feedstuff.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education and Science of the Republic of Serbia under Project TR-31037.

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THE SAFETY MANAGEMENT SYSTEM OF FOOD CHAIN

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ABSTRACT: Production and circulation of alimentary products are one of the most important activities, and food is an important category of consumer's goods. It can be said that the risks related to the food chain are present in our lives on daily basis. Therefore, health regularity is the first and unconditional request to meet every alimentary product. Healthy safe food is important and from aspect of lowering costs of population healthy treatment, whose diseases are arising from unsafe food usage. It is expected that, by introducing the safety management system of food and by food market regulation, these costs will decrease in the near future. The aim of implementation of the Standards is that, at the global level, these set of common rules will harmonize requirements for safety food management, for all of the jobs in the food chain.
System ISO 22000 sets requirements for the safety management system of food, determining necessary hygienic conditions for the production of correct and healthy safe food. By implementing standards for the safety management system of food chain, it is acting preventable, and identification of production process problems is doing. By this means enterprises get more efficient and more cost-effective protection from unwanted consequences.
Key words: *food, quality, ISO Standards 22000, satisfaction, cost-effectiveness*

INTRODUCTION

Food products are bought and spent for their biochemical values, necessary for the development, maintenance, and daily activities. Its basic function is to satisfy biological needs of human organism. Food consumption is continuous, because of that purchase of food may not be delayed. Having in mind the importance of food in the life of men, it is quite natural that there is a continued need to provide, not only sufficient quantities, but above all, sufficient quantities of totally safe food. In the process of food offering participate individual producers and companies from the field of agricultural production, industrial processing, trade, and all of they can influence to the security of products.
Health problems related to food and the diet show the tendency of growth (Henry Bolton et. al, 2008). Frequency of showing analysis of infectious disease caused by microrganisms in the world has shown that the irregular poisoning is most often a result of treatment in the course of food preparation (Egan et. al, 2007).
Food accuracy depends on processes, which are applied in the primary production of agricultural products, their industrial processing, packaging and manipulation. Food contamination sources may be auxiliary raw materials, packaging, package material, a tool for maintaining hygiene and disinfection, as well as herbicides and pesticides.
Also, foul products can occur and during its non-responsive storage, as well as during the direct use in the household (Milovanović, 2011). For these reasons, as the consequence of different forms of contamination, health and/or safety of consumers may be affected.
Because of occurrence of food crisis in Europe, the quality and food safety have become a significant topic. Food Industry, as one of the most sensitive areas with aspect to the microbiological contamination, deserves particular attention. For these reasons, must be introduced in practice new skills in the management of safety of food „from fields to the table".
It is needful to master the field of food safety through good practice at the different levels of production, distribution and consumption. Parameters that enter in a chain of supply food, as

the new resources, new processes and technology, as a new ways of representation, are the key factors for the construction of a new dimension in the area of food security, which must be managed.
In an effort to ensure food integrity and to increase security of users, legal obligations in all developed countries obliges, and development programs of food producers predict, planned introduction of the system of management.
Given to the fact that Serbia has all of the conditions for the production and export of high-quality and safe food, it is necessary to intensify in the food industry implementation and development of the management system production process of food as goods.

CRITERIONS FOR QUALITY DEFINITON

The role in establishing a system of quality management is very important. It is an important at:

- Determining access to the establishment of quality system
- Proving lines of arguments for the development and improvement of quality systems and
- During the realization of it.

In order to be accepted system of management of quality, primarily should be applied a strategic decision-making. On design and implementation of specific type of quality management, affect different needs, defined goals, provided product or provided services, all activities, as well as the size and structure of the organization. For this reason it is almost impossible to expect community in the structure of quality management.
Main aspects for development of quality management denote next phases:

- Inspection - sorting according to quality ,
- Quality control - planning control ,
- Quality assurance - identifying consumer needs and
- Other activities whose basis is a management of quality - improving effectiveness makes business development

Keeping in mind all bigger complexity of business and environment, the success of organization may result from the use and maintenance of quality management system which is designed so that permanently improves performance of system, process, staff and products, gathering needs of all interested parties,
Standard includes eight quality management principles which should be respected in process of guiding organizations, in order to improve performance.

- The guidance provided to all users,
- Management ,
- Full involvement of staff,
- The systematic approach to management,
- The continued improvement,
- Decision making based on data analysis and information received,
- The mutually beneficial relations and
- Access to the process.

Once established quality management system, must continuously improve its effectiveness (just with responsible management personnel and management of resources). In order to transform input elements and manage its processes, system must define and provide human resource, infrastructure and the working environment.
Also, the products realization, as well as and the process of measurement, analysis and improvement, effects the quality management system effectiveness.

QUALUTY SYSTEM

The International Organization for Standardization (ISO), which is a sort of network of national institutes (162 countries) for standardization, one member from each state, with the centre of co-ordination in Geneva, is the largest world organization, which is involved with the development and issuance of international standards.
ISO standards are the technical standards that ensure technology compatibility box in the entire world. They are designed to be important and useful in the world. Standard ISO 9000 refers to quality management. This means that the organization must:

- Meet the quality requirements of customers,
- Respect legal regulations,
- constantly improving satisfaction of customers and
- Continuously improving performance.

General requirements of ISO 9000 standards are related to the obligation of organization that must establish, to document, implement and maintain system of quality management and to constantly improve its effectiveness and efficiency.
Two significant changes in the new standard ISO 9001 are related to:

- Inclusion of environment of organizations in designing and developing system of quality management in the organization and
- Request that staffs that performs tasks, which affect the compliance with the requirements, must be competent in terms of education, training, knowledge and experience.

Considering the numerous opportunities for food contamination, and above all consequences to the health and safety of people, it can be presumed that the concept of risk analysis and critical control points (HACCP), which is defined by *Codex Alimentarius* for identification, evaluation and control, System of signalization of significant importance for food safety, helps producers to produce healthy food.
The core of the HACCP concept of management system is contained in constant effort that through concrete planned activities, anticipate and define all phases or situation in the entire production cycle that could lead to danger of human health or to endanger safety of consumers.
The concept of HACCP is very significant, because it allows monitoring all production phase, so based on production documentation at any time it can be determined an executor of certain technological grips, which means that it can determine and direct responsibility for security products (figure 1).

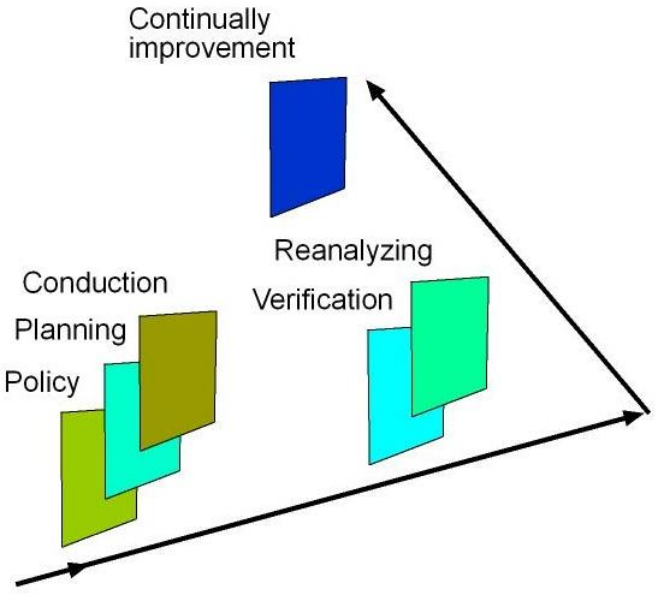

Figure 1. Model of responsibility

HACCP system of governance is not only a necessary condition for the production and export products, but is responsible and for a safe sale. A growing number trade chains operating in Serbia require from the manufacturer to its technological processes adapted with HACCP concept.
Health system guarantees to consumer a safe food, which, also positively influence the competitiveness of products and producers. Therefore, respect for and implementation of mentioned system is what will be required and in the European Union, as well as and on the BRIK market.

UINFLUENCE THAT HAVE MARKETING IN INTEGRATED SYSTEM OF CONTROL

Marketing of healthy and safe food contribute facilitation of natural conditions for the development of healthy agricultural production and processing, encourages implementation of the healthy food consumers and affect the development of the market of healthy food.
By means of marketing campaign should insist on nutrition and low caloric values of food, on its healthy component. Also, it must be emphasized the use of healthy technologies in the production, processing and retail.
In the process of transformation, producers do not need to apply technological procedures which enhance organoleptic characteristics of processing material using synthetic preparations. Quality of healthy food must be sustainable, and health-safety value preserved in processing process.
The role of marketing can be seen from the point of support the development of new technological procedures of preservation, storage and transport of sensitive health food. Marketing participate in finding adequate financial support for the development of new technologies and consequently new products of modern design and contents.
Also, the task of marketing is, that with the help of educational institutions, participate in professional development of all participants in the development, production, placement and quality control of healthy food.
In the terms of stepped-up competition in national, as well as and on the world market, companies are fighting for retention of leadership position, or for conquering new markets. One of the ways that the company battles with problems globalisation is the use logistics in order to provide an advantage in relation to competitors.
The basic logistics task as business functions is the optimization of flow of materials, information and services from entering the system to exiting from it. In order to achieve set task, logistics must use, a complicated mathematical-statistical procedure, support for an integrated information system and high-quality and professional management.
The International Organization for standardization has developed a ISO 28000:2007 specification for the Safety management system in the chain of supply. This standard requires from organization to determine whether adequate security measures are applied and to identify the legal requirements that must be align with it. If some security needs are identified, organization must implement mechanisms and processes which meet the needs.
Risk in the chain of supply is an event that may damage the organization, destroy equipment or goods, or prevent production and delivery.
Standard ISO 28000:2007 asks from companies to:

- Defines the risks that were discovered in the chain of supply through plan security,
- Create report on vulnerabilities of chain of supply in order to define safety scenario,
- Identify critical points and
- Develop a training program.

By introducing the standards ISO 28000, organization sends a clear signal to partners and clients that is a serious participant in the chain of supply, who took concrete steps in order to ensure the flow of material to be safe and effective.

CONCLUSIONS

In basis of the concept of integrated system of control is standard ISO 22000:2005. Standard is a combination of HACCP system and elements of standard ISO 9001:2000 for quality management system. Significant features of the process are its cover of complete alimentary chain.
The core of standard is following elements:

- The interactive communication,
- The system access,
- The following program and
- Principles of HACCP system.

Producers can overcome risks of technological process, and in that way guarantee food safety with their successful functioning. Significant place in the structure of system concept belongs to risk analysis, which provides a high level of protection of human health and life.
The risk analysis includes three interconnected elements:

- The risk assessment,
- The risk management and
- Transfer of information about risk.

In the part in which defines pollutants from living environment and on-farm, risk assessment and risk management implement ecologist and veterinarians.
Significant aspects in primary production may be:

- Implementation of general principles of food hygiene and control,
- Program of permanent supervision of animal health situation and
- Evaluation procedures of different risk and internal validation in accordance with HACCP system.

Obtained information is important, because on the basis of their assessment is carried out a certain technological procedures. These information, also become a part documentation which is base for the health card, which tracks each product.
Also, an important part of the concept is risk analysis and critical control point related to technological processes. Presented area includes the following:

- The determination of control points according to the production process,
- Definition of optimal values and
- Control.

Therefore, completion of the system is a management of safety in food production, which means constantly process monitoring, from entering raw materials, handling, production, distribution and consumption of the final products, and functional through analysis and control of biological, chemical and physical hazards.

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THE STUDY OF SUPPLY AND DEMAND OF ORGANIC PRODUCTS IN THE EUROPEAN UNION AND SERBIA

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ABSTRACT: One of the phenomenons that marked twentieth century is rapid technology progress. This process did not avoid food production and processing. Thanks to rapid development of technology, there has been a development of conventional food production, as well as appearance of genetically modified food. As a consequence of this trend, as well as the concern among some part of human population for their health, we had the return to natural food production in the form of the development of organic food production. One of the characteristics of organic food market in the European Union is the constant growth of demand for this product group, and insufficient quantities of organic products available in this market. These information led to the conclusion that organic food represents an opportunity for less developed countries, i.e. that countries like Serbia can manufacture and sale these products on the market with the highest purchasing power in the world - the European Union market. In this paper the characteristics of demand for organic foods in the European Union where presented, then it was explained in detail why are these products export chance of Serbia, as well as reasons why is the export of organic food from Serbia to the EU very low at the time being. At the end some recommendations that could help Serbia to increase export of organic product to EU countries were proposed.
Key words: *organic products, supply of organic products, supply for organic products in EU*

INTRODUCTION

The technological revolution that started in the twentieth and resumed in the twenty - first century did not pass the food products market. Bearing in mind the constant population growth on one side and limited amount of the arable land on the other, mankind found a new challenge - how to provide food for this ever increasing world population. In order to solve this problem new technologies in food production have been developed and their goal was to achieve increased yield per hectare (Sudarevic 1999). That was the reason why people started using large number of chemical substances throughout the twentieth century, such as pesticides and fertilizers in order to improve efficiency of food production. The research went one step further and made the production of genetically modified food possible. This type of production includes changing of the genetic make up of an organism in order to increase its useful properties such as weight, size or resistance to various diseases.

This trend has twofold consequences. The first consequence of it is the fact that the yield of agricultural crops over the past hundred years or so has increased several times. For example, according to the *CIMMYT* - International Maize and Wheat Improvement Center (2011), crop production increased three times on the same amount of land over the period 1951 to 1991. In that way possible food shortages around the world have been avoided. The second consequence is related to health challenges in the production of conventional and genetically modified food. Even though, because of the methodological requirements, it is extremely hard to prove that both conventional and genetically modified food can be harmful to human health. Today a growing number of consumers believe that the food produced in this way can have adverse consequences.

There is a new methodology of food production as a response to the problems we stated – it is called organic farming. There are several definitions of organic food. According to Allen and Albala (2007) Organic food represents food produced using methods which don't include

modern synthetic pesticides, chemical fertilizers, and which don't include genetically modified food, the food produced without the use of radiation, industrial solvents or chemical food additives. According to International Federation of Organic Agriculture Movements (2011) organic farming is a food production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects Organic Agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved. We can conclude from these two observations that this type of production is based on the natural way of producing food without addition of any artificial substances and by respecting the principles of health, ecology and equity concerns.
Starting with the stated problems perceived by consumers when it comes to consumption of conventionally produced agricultural products, demand for organic agricultural products is on the rise in market-income countries, especially in most EU member states.
On the other hand, there is not enough unpolluted soil suitable for this type of production so we can conclude that the possibilities of producing organic agricultural products are limited. The only possible solution to meet consumers needs on given markets, at the moment (and in the near future) would be the import of organic products. Organic food market in the European Union is one of the rare markets where demand consistently exceeds supply which makes it extremely attractive to bidders from different countries. Hence the optimism that one of the opportunities for the development of Serbian economy lies in the right investment in both production and export of organic food.
The purpose of this study is to explore the prospects of the export of organic products from Serbia to the markets of developed countries, primarily the European Union.

MACURRENT STATE OF DEMAND FOR ORGANIC FOOD IN THE EU

When someone should describe an ideal market in which he/she wishes to perform, that person would almost certainly state the two such market characteristics: the fact that demand exceeds supply and that market is poised for continued stable and predictable growth (Kotler 2011). Organic food market is one of the few in which the demand for products exceeds supply and in which we have a constant stable growth over the years (European Commission, 2011). According to the EU report about the organic sector, organic food market for the EU-15 countries (members which joined the Union before 2004) in 2006/2007 amounted to EUR 14.4 billion. Up to 80% of that amount (around EUR 11.5 billion) goes to four largest consumers - Germany, United Kingdom, France and Italy. When it comes to relative amounts, the consumption was highest in Austria (around 5%), followed by Germany, Denmark and Luxembourg (3.7% /3.8%). What we can notice from the report is the significantly lower demand for organic products in the EU -12 countries (countries which joined the EU in 2004, together with Bulgaria and Romania). In these countries demand for these sort of products is less than 0.2%, while only in Czech Republic (0.5%) it is higher than the total demand for food products.
Another interesting trend is the significant growth of organic food in the EU over the last ten years. According to the same report there has been a growth of demand for organic food of 18.1% in France for the period 2005 to 2009, 14.0% in Germany for the period 2000-2008, 8.7% in Italy for the period 2001-2009, and 11.9% in United Kingdom for the period 2000-2008. It is interesting that the economic crises caused a drop in demand for organic food only in the UK, while it recorded a growth in Germany, France and Italy.
The report also reveals the fact that the demand for organic food exceeds supply. As a result of this trend we can come to the conclusion that the majority of member states will be forced to import organic food in order to solve this problem. It is expected that a trade between members and non-members of EU in the field of organic food will record a significant increase in the future thus solving the problem of lacking these types of products.
In addition to this report we can find the research in organic food conducted by Richter and Padel (Organic Eprints, 2011). The results of their research are presented in the chart 4. Results are related to the European countries in 2005.

Table 1. Demand for organic food in Europe for 2005 (Organic Eprints, 2011)

Country	Organic market in billions of euros	Market growth percentages compared to 2004	Consumption per capita in euros (€)	Market share of organic food compared to conventional food
Germany	3.9	11%	47	around 3%
Italy	2.4	-----	42	-----
Great Britain	2.33	30%	39	-----
France	2.2	-----	37	-----
Denmark	0.307	10%	57	-----
Austria	0.450	12%	56	around 3%
Holland	0.419	1.4	25	-----
Spain	0.300	-----	7	-----
Switzerland	0.763	-1%	103	around 4.5%
Norway	0.041	-----	10.2	-----
Czech Republic	0.012	30%	1.14	-----

Research by Richter and Padel confirms analysis of the European Union that the organic food market is relatively large with tendency of growth. Market of the four largest countries in the European Union for 2005 exceeds 10 million euros. The biggest market in Europe is Germany with 3.9 billion euros, followed by Italy, France and United Kingdom with over 2 billion euros each. What is interesting is that the United Kingdom achieved highest rate of market growth for the period 2004-2005, with over 30% which might explain the decline in demand for organic foods during the crisis. Switzerland has the highest consumption of organic food per capita by far, it amounts to 103 euros which is more than twice the one of Denmark and Austria, the countries with highest consumption of organic food per capita within the European Union. This data shows that Switzerland, although the market outside the European Union, should not be neglected.

ORGANIC FOOD IN SERBIA

In the remainder of this paper, we stated the main advantages of production and export of organic food from Serbia, followed by the current status of organic food production in Serbia, and finally the factors that negatively affect the current balance of exports from Serbia to European Union.

Organic food as an export opportunity for Serbia

There are several important reasons why organic food represents great opportunity for the development of Serbian economy. They are reflected in cheap labor, large amount of arable land, proximity to the markets of the European Union, and high demand for organic products.

Cheap labor. The way of production and processing of organic food are the reason why this type of production is considered to be labor-intensive industry, that is to say, in order to obtain the same amount of output for the production of organic food it is necessary to employ more labor than conventional food production. This is considered to be one of the primary reasons why organic food is more expensive than conventional food. Since the average salary in Serbia is several times lower than the average salary in certain European countries (according to the Bureau of Statistics average net wage salary in Serbia currently amounts to over RSD 38000 (around EUR 380) (Statistical Office of the Republic of Serbia, 2011), we can say that the production of organic food is far more profitable in Serbia than in EU countries. The unemployment rates are among the highest in Europe (around 22%), so we can conclude that the production of organic food could be great opportunity to launch Serbian economy and reduce unemployment.

A Large Amount of Arable Land - One of the main characteristics of Serbia are large areas of arable and fertile land. The soil fertility makes it possible to produce large amounts of food

without the addition of synthetic inputs such as fertilizers and pesticides. On the other hand, the soil diversity makes it possible to grow various types of crops. This is very important because it is necessary to round up the production process in order for organic production to take place, it is not enough to produce only one crop (Figure 1).

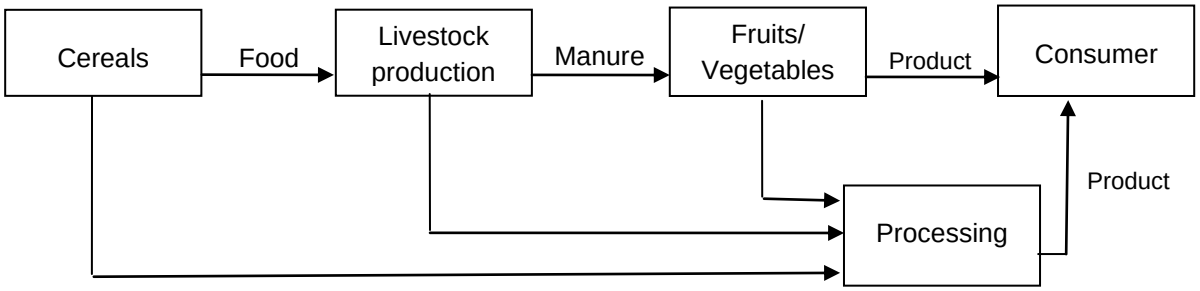

Figure 1. Possible alternatives to organic food production

So for example, after grains have been processed they can be used for direct sale at the market (flour, cornflakes, *etc*.) or they can be used as an ingredient in animal feed. The benefits of livestock production in an organic way may be multiply beneficial: after meat have been processed it can be marketed, it is possible to produce milk and milk products, eggs as well, which can be placed on the final consumers market. Manure can also be used for fertilizing fields in order to get higher yields from organic fruits, vegetables and grains. Organic fruits and vegetables can be sold directly on the market, but can be processed as well (juice, jam, salad in a jar, *etc*.), and then can be placed on the market. Organic food makes sense only if all listed manufacturing segments are covered, which can best be seen on the example of Austria, which achieves the best results in the production of organic food in Europe. In addition, the land in Serbia is still largely preserved for organic production (Sudarevic et all 2011) because of poor use of pesticides and fertilizers due to farmer's low purchasing power, which is not common in most EU countries.

Proximity to EU markets - Another advantage for Serbia could be the fact that it is close to many EU markets, primarily Austria, Italy, Germany and even France, so the transportation costs wouldn't be high and there would be more time to sell the goods, especially if we take into consideration the short-term duration of most food products. The duty-free agreement between Serbia and European Union allows export with no additional export taxes.

High Demand for Organic Products - As it was stated in the previous part of the study, demand for this group of products goes beyond the production possibility, and is poised for continued growth, so we can assume that this trend will continue. We can therefore conclude that the organic food market is a very attractive market.
After defining the factors that are contributing to the development of organic food in Serbia we are going to describe the current state of this agricultural sector in Serbia.

The Limiting Factors
Given the chances that organic food production carries, but a small area of land in Serbia on which this type of production is being used right now as well, it is necessary to define the specific reasons for modest production level of organic food in Serbia. The authors made a list of the limiting factors affecting the level of production of organic food in Serbia by reviewing the literature and by interviewing the experts in the field of organic production.

High Capital Costs - One of the characteristics of the organic production is the fact that it is necessary to have higher initial investment compared to conventional production. This investment can later be compensated by higher prices for the organic products. In Serbia, the problem lies in the fact that capital is very expensive, often several times more expensive compared to the countries of the European Union, which makes the manufacturing process

more expensive as well. Also, banks in Serbia require a high degree of collateral, often in the form of mortgages and / or guarantors, so only few people in Serbia decide to enter into any risky venture because miscalculation may lead to loss of home.
Many estates in Serbia are very small and it often happens that the land of the farmers is located in several locations, which further complicates the manufacturing process (Gulan, B., 2011).
If one considers that organic farming to be labor-intensive, fragmentation of arable land in several locations that each household has, can be a serious problem. On the other hand, many farmers produce the same crops over the years, and even generations back, in more or less the same way, possibly with some technological advances, and that is why they are not willing to experiment with new varieties and new production methods.

Lack of training - Although organic farming is nothing new for the experts, this type of production represents the great unknown. What needs to be done to increase the volume of organic production is a general education of the producers on both the benefits and characteristics of organic farming. It is realistic to expect that the rapprochement between scientific research institutions and manufacturers will lead to increased production of organic products in Serbia. One way of solving this problem might be the employment of a large number of unemployed agronomists who would receive salary from the state which would result in the farmers education. Of course, education about organic food must be a priority in the education of Serbian farmers.

Lack of Variety - According to some authors (Curić J., Ceranić S., 2010) another big problem is a lack of suitable varieties for organic production. This type of production requires the return of some forgotten varieties which posses far better characteristics for this type of production, in relation to available, "modern" varieties. The trouble is that it is very difficult to get those varieties in Serbia due to low demand for these sorts among the existing manufacturers. The development of this market is sure to find a motive for the production and import of some varieties that are missing.

Low Demand for Organic Products in Serbia. Demand for organic products in Serbia is low and we can say that this market is still in its infancy. It is extremely hard to find organic food and the price is far higher than the price of conventional food, especially if take into consideration prices in European Union. Logical development of any company is to become a stable company in the domestic market and then continue to grow its global market performance.
Modest demand for organic food in Serbia can be one of the reasons why there has not been a large enough company that could successfully perform in the EU market. The solution to this problem could be the association of producers and sellers of organic food in Serbia which would educate the citizens of Serbia and generate the primary demand for organic products. Something like this would enable them to increase sales, and to build a cooperation as well, and in that way welcome the joint appearance on third markets .
In regards to the specific data it is very difficult to find the exact data from secondary sources on the demand for organic products in the Serbia. In this study, as a source of data was used a study *"Status and prospects of consumption of organic products"* (Center for Organic Production Selenča, 2011). The advantage of this study is that it was done in 2010, so the data can be considered relevant, while the main disadvantage is the small number of respondents, only 200 of them. The study was conducted exclusively in Novi Sad which can be another drawback, however, taking into account that it is the urban population that is primarily interested in organic food in Serbia, this research can be a solid indicator of demand for the domestic organic market. The survey (Center for Organic Production Selenča, 2011) showed that 89% of respondents is familiar with the concept of organic food. On the other hand, 14% of respondents regularly buy organic products, while 38% of respondents sometimes or rarely purchase organic foods. 48% of respondents do not buy organic products at all. As the main motive for buying organic food (Center for Organic

Production Selenča 2011) are cited health reasons (50% of respondents), then the quality of food (32% of respondents), environmental care is the third most important reason for buying these types of products (15% of respondents), and 3% of respondents did not state the reason for purchasing organic food.

Lack of Security in the Marketing of Organic Agricultural Products - The leading producers of organic agricultural products are, as a rule, the earlier highly successful farmers of conventional agricultural products. It only indicates that the success in this type of agriculture requires systematic, hard work and willingness to learn. Despite all of this, often the biggest problem for transition to this type of production (or the existence) is the possibility of exporting products at prices that are reasonable given the increased costs and reduced yields. Therefore, as a necessary condition appears the requirement that organic farmers provide the appropriate conditions of purchase by the cooperative association, or under contract with the already proven organizers of production for export to international markets.

CONCLUSIONS

Conclusion of the complete study could fit in one sentence - taking into account the characteristics of the organic food market in the European Union and the characteristics of Serbia regarding the production - possibility when it comes to this type of products, export of organic food from Serbia to the European Union would have to be much higher.
The question arises, what to do next? What is necessary to do in order to improve the situation? There are two groups of subjects that can facilitate the process in the positive direction: producers and the state. The first thing that manufacturers should do is to form the association in order to approach the EU market together, but also to generate a common deterrent demand for organic products in Serbia. As far as joint appearance is concerned, the chronic problem of Serbian farmers to meet the demands of distributors in the EU markets is already known (but not in terms of quality but in terms of quantity instead). It often happens that foreign traders find a quality product in Serbia, but later realize that manufacturers not nearly meet their needs. This problem could be solved by manufacturers association, but also by focusing only on certain varieties that would have the best pass and/or which would wear the highest return on invested resources. Association would make it possible to generate greater demand for the organic products in Serbia.
The state can help the manufacturers with both financial and non financial measures. As regards the financial measures, providing strong subsidies for production of certain crops or animals under organic production can positively influence farmers to adopt this form of food production. In cooperation with commercial banks, it is possible to offer more favorable loans where the state would be taking on given cost (interest) or accepting at least a part of the risk if the loans are not returned. These activities would lead to a spillover of risks from the manufacturer to the state which might have beneficial effects on the increase of the organic production. Another direction would certainly be the education of agricultural holdings on the use and characteristics of organic food production. This could be done by linking scientific research institutions and the farms together. On the other hand, creating certain institutions that would help sales of organic food would certainly help both existing and potential producers of these types of products. Also, import of certain varieties that are suitable for organic production and which cannot be found on domestic market would be much easier if certain ministries participated in the enterprise. The state made the first step in hiring agronomists and in providing subsidies to the producers of organic food, but the question is whether this is enough in order to significantly increase production and export of this type of products.

ACKNOWLEDGEMENTS

The author(s) gratefully acknowledge financial support from the Ministry of Education and Science of the Republic of Serbia (Project III 46005).

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THE SPECIFICS OF THE HACCP SYSTEM APPLICATION IN HONEY PRODUCTION

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ABSTRACT: The purpose of this paper is to present the implementation of the HACCP system, as a system which ensures the product safety, in the field of honey production of the Primorje-Gorski Kotar County agricultural cooperative which engages in production of its own honey. The implementation of the HACCP system in honey production is presented through the process steps, the risk analysis, and the using of methodology for the detection of critical control points (CCPs) and control measures, which resulted in the HACCP plan design. The flowchart presents 24 process steps, 4 of which demand the control measures to be carried out to secure the food safety. During the implementation of the HACCP system, all the elements of prerequisite programs for good production and hygienic practice were taken into consideration. In the Republic of Croatia, honey and other bee products satisfy quantitative and qualitative domestic market.
Key words: *honey; HACCP; food safety*

INTRODUCTION

Gradual change of orientation of agricultural policies towards sustainable and integrated agricultural development through concepts and instruments of multifunctional development at the same time presents a chance for the development of beekeeping in the Republic of Croatia. Thus the purpose of this paper is to present the implementation of the HACCP system, as a system which ensures the product safety, in the field of honey production of the Primorje-Gorski Kotar County agricultural cooperative which engages in production of its own honey. The activity of the cooperative is based on the production of their own honey, buying off honey, honey production and honey products, and the sale of these products. This includes the production of catering packaging of honey or packaging of honey in plastic containers of 20 grams and glass jars of 25 grams. The capacity of honey processing amounts to a maximum of 10 tons per year.
The implementation of the HACCP system was preceded by the establishment of the HACCP team (members, duties of members, duties of employees), fulfillment of prerequisites of the program of good hygienic and good manufacturing practices (cleaning and disinfection, hygiene, training, equipment maintenance, requirements for visitors, etc.), making the product description (composition, characteristics, packaging, packing, storage conditions, transport, and shelf life), defining the process of traceability, product withdrawal and recall, as well as customer complaints (MPRRR, 2007).

MATERIAL AND METHODS

The implementation of the HACCP system in honey production – in jars and catering packaging - is presented through the process steps, the risk analysis, and the using of methodology for the detection of critical control points (CCPs) and control measures, which resulted in the HACCP plan design.
Process steps show the steps (phases) in the honey production in glass containers and catering packaging.

A HACCP study was made. It comprised a description of the physical, chemical and biological hazards in the materials' receipt, manufacturing, packaging, storing, transporting and selling honey and honey products, which represents risk analysis.
The methodology used for the application of the detection of critical control points (CCPs) is – "Decision Tree" – Figure 1, as well as the application of the methodology for the implementation of control measures.

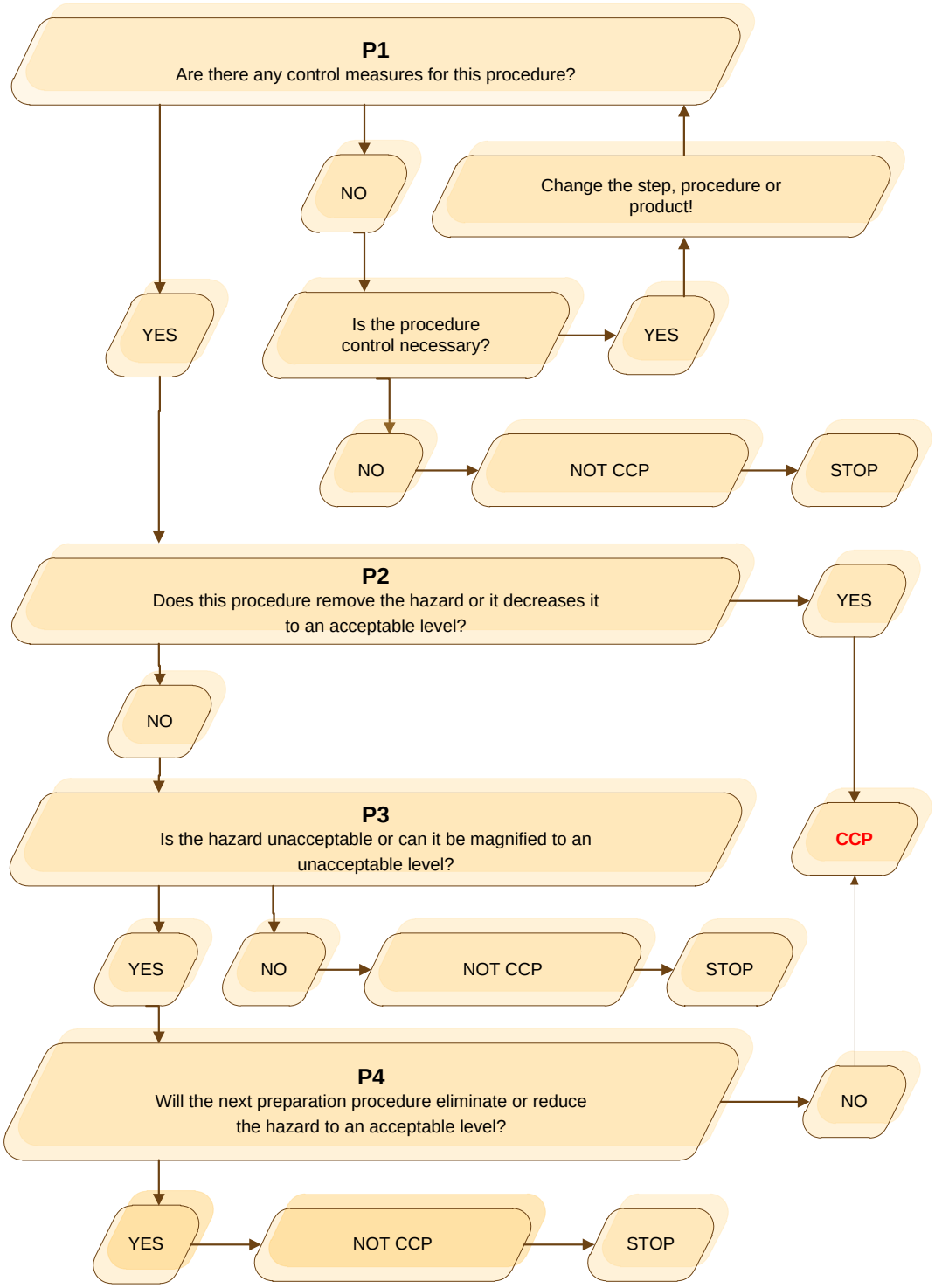

Figure 1. Decision Tree

RESULTS AND DISCUSSION

The flowchart presents 24 process steps, 4 of which demand the control measures to be carried out to secure the food safety (Figure 2).

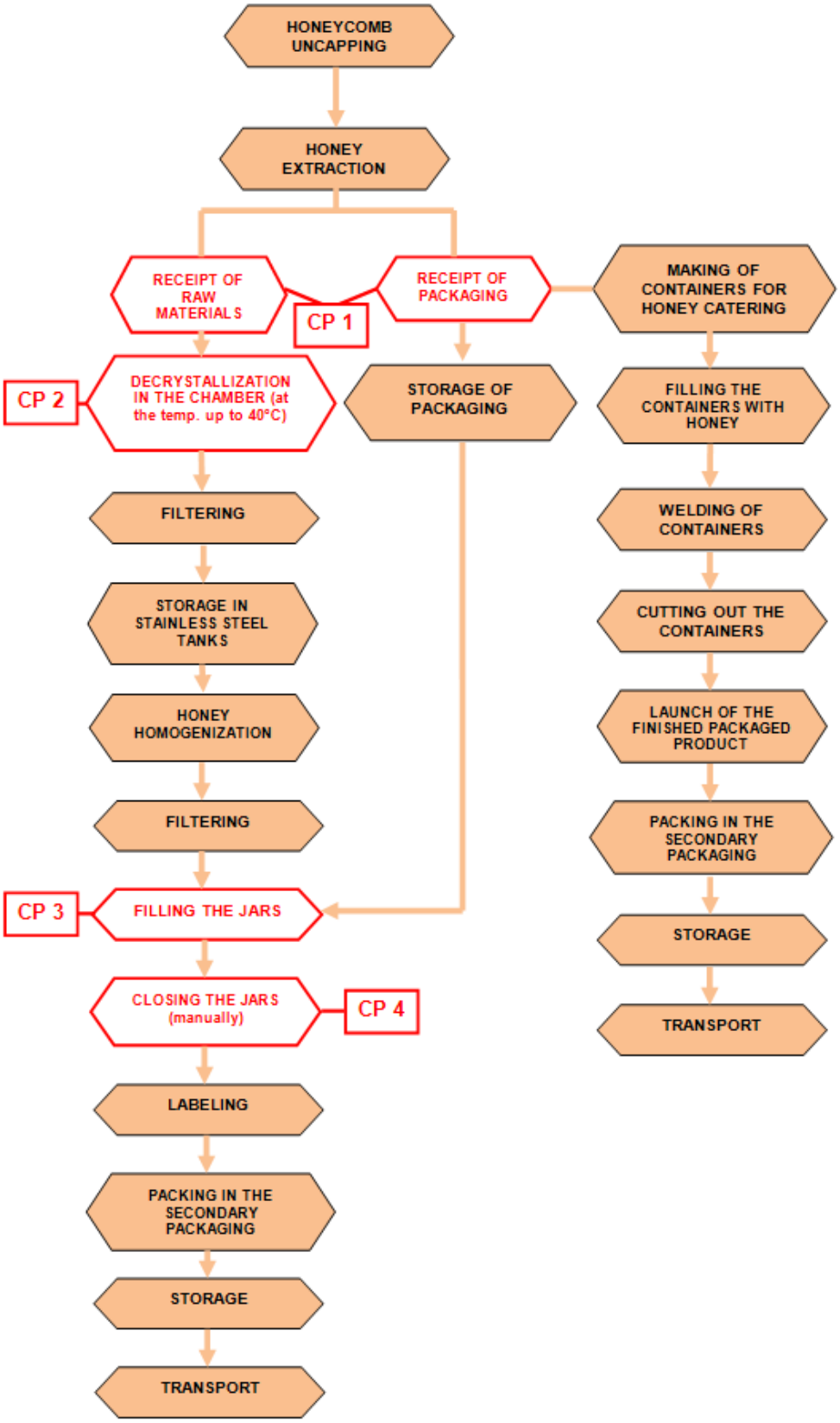

Figure 2. Flowchart

The methodology presented used for the determination of CCP-s and control measures takes into consideration the probability of risks and the amount of danger. The HACCP plan presents the type and description of indentified risk, critical limits, corrective measures and the way of implementing monitoring through procedures, frequency and responsibility, for only 4 process steps, which present the implementation of the control measures: raw materials receipt, packaging receipt, jar filling and jar closing (Tables 1-3).

Tables 1-3. HACCP plan

PROCEDURE IN THE PROCESS	POTENTIAL RISK	P1	P2	P3	P4	CP/CCP	CONTROL MEASURE
3 and 4 RECEIPT OF RAW MATERIALS / PACKAGING	**PH:**	**YES**	**NO**	**NO**		**CP 1**	- safety of raw materials/packaging - possibility of removing contamination by later procedures - employee training for the receipt of raw materials/packaging
	Presence of physical contamination due to damage and unclean packaging.						
	CH:	**YES**	**NO**	**YES**	**YES**	**CP 1**	- safety of raw materials – analytical reports for raw materials - safety of packaging – analytical reports and/or certificates for the purpose of packaging in food industry - employee training for the receipt of raw materials/packaging
	Presence of toxins, chemical substances from the environment, food additives and residues of pesticides.						
	B/M:	**YES**	**NO**	**YES**	**YES**	**CP 1**	- safety of raw materials – analytical reports for raw materials - safety of packaging – analytical reports and/or certificates for the purpose of packaging in food industry - employee training for the receipt of raw materials/packaging
	Presence of microorganisms (moulds, bacteria, and viruses) and spores.						

PROCEDURE IN THE PROCESS	POTENTIAL RISK	P1	P2	P3	P4	CP/CCP	CONTROL MEASURE
9 JAR FILLING	**PH:**	NO	NO			low risk	- GPP (employee training, preventive equipment maintenance) - GHP (SSOP, personal hygiene, employee training)
	Contamination by physical damage (equipment, hands of employees, place).						
	CH:	YES	NO	YES	YES	low risk	- GPP (employee training) - GHP (SSOP, employee training) - quality guarantee of the packaging supplier - supporting documents (analytical reports)
	Contamination by cleaners and disinfection. Contamination by chemical substances from the packaging.						
	B/M:	YES	NO	YES	YES	low risk	**-** GPP (employee training) - GHP (SSOP, employees' hygiene, employee training) - quality guarantee of the packaging supplier
	Contamination by microorganisms from the place, hands of employees, equipment, and packaging.						

PROCEDURE IN THE PROCESS	POTENTIAL RISK	P1	P2	P3	P4	CP/CCP	CONTROL MEASURE
10 JAR CLOSING (manually)	**PH:**	YES	NO	NO		**CP 2**	- GPP (employee training) - GHP (SSOP, employee training)
	Physical contamination due to irregular procedure of jar closing.						
	CH:	YES	NO	YES	YES	**CP 2**	- GPP (employee training), - GHP (SSOP, employee training) - quality guarantee of the packaging supplier - supporting documents (analytical reports) - visual control of closing
	Contamination by cleaners and disinfection. Contamination by chemical substances from the packaging. **Chemical contamination due to irregular procedure of jar closing**.						
	B/M:	YES	NO	YES	YES	**CP 2**	**-** GPP (employee training), - GHP (SSOP, employee training), - visual control of jar closing
	Contamination by microorganisms due to irregular procedure of jar closing. **Contamination by spores of microorganisms due to irregular procedure of jar closing.** **Growth of microorganisms due to irregular procedure of jar closing.** **Germination of spores due to irregular procedure of jar closing**.						

CONCLUSIONS

During the implementation of the HACCP system, all the elements of prerequisite programs for good production and hygienic practice were taken into consideration.
Honey, because of its intrinsic characteristics, especially the sugar content, represents a rewarding type of food when it comes to ensuring safety.
The HACCP plan defines 4 process steps in the honey production in jars and catering packaging which demand implementation of control measures for ensuring the product safety. These are as follows: raw materials receipt, packaging receipt, jar filling and jar closing. In the Republic of Croatia, honey and other bee products satisfy quantitative and qualitative domestic market.

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TYPICAL AGRICULTURAL PRODUCTS AS A WAY OF DIVERSIFICATION OF RURAL ECONOMY IN BOSNIA AND HERZEGOVINA – CASE STUDY OF GRAH POLJAK

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ABSTRACT: Bosnia and Herzegovina (BiH) is a small country whose agricultural production in the current moment of dominant globalization cannot be competitive in terms of quantity, nor from the point of application of new technologies in agricultural production. Perhaps the best opportunity for the agricultural sector of BiH lies in the revitalization of traditional production technologies whose application leads to very high quality products and level of recognition in a broader context. Typical agri-food products diversify rural economy, contributing to the reduction in unemployment of rural population and provide greater employment opportunities for vulnerable rural populations. These products provide a developmental perspective to small farms to better valorize their potential. Valorization of local, traditional agri-food products is a complex process that involves a large number of stakeholders, who have to adjust their goals and values. There are number of agri-food products in BiH that can be characterized as typical products. Among the three products international organization Slow Food recognized as typical in BiH, this paper analyses *grah (bean) poljak* from Petrovo polje - the municipality of Trebinje, which has the Ark of Taste status. Based on data collected from producers in the field, it is found that the profitability of production of *grah poljak* as a typical product, is much higher (profit of EUR 8,917 per ha) compared to conventional cultivation of beans (profit of EUR 3,329 per ha). Therefore, production of *grah poljak* is more than profitable and main barrier to this production may be some other elements of the entire production cycle. This primarily refers to the sale and complete implementation of other marketing elements (distribution and promotion).
Key words: *typical agricultural products, diversification, rural economy, grah poljak*

INTRODUCTION

Rural development policy in Europe has been recognized as a means of preserving the natural and traditional values, which helps preserve national and cultural identities of individual countries. Local, traditional or typical agricultural products and rural development are already significant and representative binomial in many EU countries which have a demonstrable potential in these sectors (Brunori, 2006). Development of legislation and implementation of EU policies on rural development and qualification of production have made that specifics of agricultural and food products become prominent. On the one hand, these specifics are reflected in diversification of rural economy and on the other hand they are significant source of additional revenue for producers and they make the local territory recognizable.

Typical agricultural product can be defined as a product characterized by unique quality attributes that reflect a specifics of certain territorial environment (Brunori and Pacciani, 2006). Characteristics and quality of products are therefore impossible to reproduce in other places, or outside a particular territorial and economic environment. It is a product obtained by implementation of traditional production methods, using specific local resources that provide specific and identifiable quality characteristics and in its title bears the name of the locality from which it comes from (Marescoti, 2006). The specific quality of a product may result from pedological-climatic ambient or from the specifics of manufacturing processes.

Typical agricultural product is the result of collective and localized historical process, accumulated knowledge and skills that are based on a combination of territorial resources, both physical and anthropological. (Pacciani et al., 2001, Brunori, 2007). These products represent a cultural and historical heritage of an area. Since the integrated rural development is based not only on intensive agricultural production, but much more on the revitalization of forgotten and extensive production methods, this creates a new perspective for almost forgotten products to become generators of development in rural areas – one of them is *grah poljak* from Trebinje. In order to revitalize the village, one of the best methods is to preserve and revalorize traditional products of high value.

Bosnia and Herzegovina is a small country whose agricultural production in the current moment of dominant globalization cannot be competitive in terms of quantity, nor from the point of application of new technologies in agricultural production (Samardžić, 2009). Perhaps the best opportunity for the agricultural sector of BiH lies in the revitalization of traditional production technologies whose application leads to very high quality products and level of recognition in a broader context. (Bajramović and Samardžić, 2011). There are numerous products in BiH who are identified as potential typical agri-food products (Bajramović, 2010, Čustović et al., 2009). However, the very existence of resources possessing awareness has no value without its valorization. It is long and arduous process that leads to sustainability of the stakeholders involved in the valorization of resources.

By definition, in terms of dynamic liberalization of international markets, small farms are exposed to high business risk, and their survival as an economic unit is seriously impaired. In this situation, small farms are finding out development strategies that would help them to overcome their core limitation, deficient physical capital. Activation of other resources: knowledge, skills, labor, social capital and so on, is the only development alternative for this widespread socio-economic group (Bogdanov, 2007). Small farms are in a constant pursuit of finding nontraditional ways to increase the added value of their products in order to stay in the race with major manufacturers[1]. One of the competitive factors of small farms is the experienced labor force, which can hardly be copied and major manufacturers cannot easily incorporate it into their production technology and market distribution. The experience, skills and specific knowledge in the production process and product handling are what small farms have as an advantage compared to large farms, and it is closely associated with typical features of products. These factors contribute to greater personalization of products, opening the door to create a development strategy for small farms.

BiH is characterized by a rich ecosystem and biodiversity, which enables the production of various agricultural products. A mixture of cultures and historical heritage of the peoples, who live in this region, is condition of presence of a large number of specific food products, with original manufacturing technology and finishing / processing (Ramić, 2011). The aim of this paper is to take *grah poljak,* certified as a typical BiH agricultural product (Slow Food Ark of Taste status) as an example of the way how to obtain the status of typical BiH agricultural product and achieved level of protection and valorization, and to recognize the economic characteristics of its production compared to the conventional method, all in light of much-needed diversification of BiH rural economy.

MATERIAL AND METHODS

This study used number of recognized scientific and professional methods of which two stand out: the analysis (review) of the relevant literature and learning from good practice (case study). First method is used because issue of typical agricultural products in BiH and diversification of rural economy in general is not elaborated enough and the second one

[1] Additional knowledge and skills of the workforce is capital evaluated relatively low by farms in BiH compared to other resources and they have insufficient knowledge how to mobilize them to generate external revenues. Most commonly seen manifestation of specific knowledge and skills, is women's art to prepare fruit and milk in a traditional manner, knowledge of handicrafts, etc.

because learning from good practice is generally accepted method to understand and introduce new forms of economic activity using the positive experiences of others in the best way. Finally, in economic analysis we used the usual method of comparison and accrual calculations, while the more important economic indicators were calculated based on generally accepted patterns.

RESULTS AND DISCUSSION

Grah poljak (Lathyrus sativus L.) along with Cheese in a sack and Požegača plum Slatko are the only three BiH products that are recognized as typical farm products by Slow Food, the umbrella organization for eco-gastronomic activities. Each product had its own distinctive way of recognizing and recognition, and about the way of *grah poljak* we can briefly say the following: in the past *grah poljak* was very present in the Trebinje region and it has nurtured many generations. Production of *grah poljak* started to phase-out slowly in nineteen sixties, when it was pushed back by more economical and more yielding varieties. A new story of *grah poljak* begins in 2005, when Italian organization UCODEP (today OXFAM Italia) comes in the Trebinje area to investigate the potential typical and traditional products from the region of Herzegovina, as part of the "Taste of Herzegovina" project. Together with the representatives of local community they explored the municipality of Trebinje and in the village of Zgonjevo located in Petrovo polje, story about *grah poljak* is found. Seeing that this product could be interesting as a potential typical product, the search for *grah poljak* has started. The whole village found just a few kilos. At first glance, this bean shows its specificity. It is irregular in shape, like the crushed stone, about 1 cm in size, similar to the color of colorful sea sand and it comes in many shades.

Photo 1. Grains of *Grah poljak*

Supported by UCODEP, *grah poljak* was presented at the fair of typical and traditional products in Turin (Italy) in 2006, under Slow Food sponsorship. It was promoted as a potential typical product from municipality Trebinje area. After being judged favorably at the fair, NGO „Centar za razvoj Hercegovine“ gathered people, potential *grah poljak* producers, and financially supported by Trebinje municipality, „Petrovo Polje“ agricultural cooperative was established. From that moment begins a different story for *grah poljak*. Until today (2011) major stakeholders in preserving this characteristic product and reproduction of its seed material until regular production were Trebinje municipality, cooperative „Petrovo polje“, UCODEP and NGO „Centar za razvoj Hercegovine". From initial few kilos in 2005, after implementing many activities and working in the field, production of seeds was significantly increased, so as a result in 2009, there was amount of 4.5 tons available for spring seeding. Today, at cooperative "Petrovo polje", there are about 50 producers of *grah poljak* who have produced about 25 tons with an average yield of 1,100 kg/ha, in strictly controlled conditions of production.

Promotional activities and valorization of *grah poljak* as a typical product from area of Trebinje municipality helped toward this product become one of the few typical products in BiH with Slow Food Ark of Taste status. Namely, based upon activities on valorization and promotion, participating at fairs of typical and traditional products such as Terra Madre in

Turin (Italy), confirmation for quality and organized work given by representatives of Slow Food after the monitoring of the Trebinje area and discussions with all major stakeholders, Slow Food has accepted the request of the producers of *grah poljak* to form Convivium (Community of "slow" food lovers and producers in a particular area) on 28.5.2009, which represents an important step in the ultimate protection and valorization of the product on the global level. Chemical analysis has been carried *grah poljak* in March 2010 at the Institute of Turin Chamber of Commerce, showed remarkable chemical composition. Among other things, 100 grams of *grah poljak* contains 26.23% of protein.

Based on data collected from producers in the field on expended materials and hours of operations in the labor and machine operation (and their prices) in the production of *grah poljak* and conventional beans, economic analysis was done at the level of production on a surface of 1 ha (Table 1). The goal of such analysis is the comparison of two ways of production and economic justification in general for the production of typical product of *grah poljak* compared to conventional production. Business success is figured out on the basis of the calculated revenues and expenses of production and identified indicators of productivity, economic efficiency and profitability of production.

Table 1. Comparative review of revenues and expenses in conventional bean production and production of typical agricultural product *grah poljak* (Surface 1 ha, in EUR)

Description	Unit	Bean Conventional production			Grah poljak Typical agricultural product		
		Quantity	Price	Amount	Quantity	Price	Amount
A) Revenues							
Bean – seed	kg	2,500	2.05	5,112.92	1,200	10.23	12,271.01
B) Expenses							
Material							
Seed	kg	100	3.32	332.34	120	10.23	1,227.10
Plant protection means	ha	1	153.39	153.39	-		-
Basic fertilizer	ha	1	153.39	153.39	-		-
Foliar fertilizer	ha	1	178.95	178.95	-		-
Bags for packing (25/1)	ha	1	102.26	102.26	1	51.13	51.13
Labor and Machinery work							
Plowing	ha	1	204.52	204.52	1	204.52	204.52
Harrowing	ha	1	102.26	102.26	1	102.26	102.26
Preparation for sowing	ha	1	51.13	51.13	1	51.13	51.13
Sowing (by machine)	ha	1	51.13	51.13			
Sowing (by hand)	ha	1	-	-	1	35.79	35.79
Protection (spraying)	ha	1	51.13	51.13	-		
Harvest (combine)	ha	1	127.82	127.82	-		
Harvest (by hand)	ha	1			1	766.94	766.94
Threshing (by machine)	ha	1	76.69	76.69	-		
Threshing (by hand)	ha	1			1	409.03	409.03
Transport to storehouse	ha	1	20.45	20.45	1	20.45	20.45
Picking of grain	ha	1	51.13	51.13	1	230.08	230.08
Cost of sales	ha	1	51.13	51.13	1	153.39	153.39
Other costs	ha	1	76.69	76.69	1	102.26	102.26
Total (B)				**1,784.41**			**3,354.08**
C) Profit (A-B)				**3,328.51**			**8,916.93**
D) Cost of production (EUR/kg)				**0.71**			**2.80**

***Source**: Own calculations; **Note**: Calculations were performed in BAM (convertible mark) and then converted into Euro (exchange rate: 1 EUR = 1.95583 BAM)*

A review of Table 1 shows that both productions are profitable, but profitability of production of *grah poljak* as a typical product (profit of EUR 8,917 per ha) was significantly higher compared to conventional production of beans as a vegetable crop (profit EUR 3,329 per ha). This major difference is primarily the result of considerably higher revenues expected in the production of *grah poljak* due to the expected high sales price (EUR 10.23 per kg). This high cost is possible due to the uniqueness and rarity of the product and it has been proven in the field. When it comes to production costs, they were significantly higher in the

production of *grah poljak* (EUR 3,354 per ha) compared to conventional cultivation of beans (EUR 1,784 per ha). This difference arises for two reasons: much higher cost of seed for *grah poljak* production (EUR 10.23 per kg) compared to conventional cultivation (seed beans EUR 3.32 per kg) and significantly higher cost of human labor, which are generally more expensive compared to work of machinery.

Table 2. Comparative review of the economic indicators of business success in the conventional production of beans and typical product *grah poljak* (Surface 1 ha)

Indicator	Beans Conventional production	Grah poljak Tipycal agricultural product
Elements of business success		
Production (kg)	2,500	1,200
Total revenues (EUR)	5,113	12,271
Total costs (EUR)	1,784	3,354
Cost of human labor (EUR)	51	1,442
Profit (EUR)	3,329	8,917
Indicators:		
Productivity		
1. Revenues/Cost of human labor	100	8.51
2. Yield/Cost of human labor	49	0.83
Economic efficiency		
3. Total Revenues/Total costs	2.87	3.66
Profitability		
4. Profit/Total cost*100 (%)	186.53	265.85
5. Profit/Total revenues*100 (%)	65.1	72.67

***Source**: Own calculations*

Table 2 gives a comparative overview of the economic indicators of business success in the conventional production of beans and production of typical product *grah poljak* (Surface 1 ha). Indicators of labor productivity are much higher (of better values) for the conventional production of beans compared to the *grah poljak*. Conventional production of beans with 1 EUR expenses for labor generates 100 EUR of revenues or the value of 1 EUR spent on labor generates the production of 49 kg of beans. In production of typical product *grah poljak* these values are much lower, so the value of 1 EUR expended for labor generates 8.51 EUR of revenues or the value of 1 EUR expended for labor yields 0.83 kg of beans. Other identified economic indicators of success are much more favorable (higher) in the production of typical product - *grah poljak*. The economic efficiency (the ratio between total revenue and total costs) is 3.66 and is significantly higher than in conventional production, where the amount of revenues is 2.87 times the amount of total costs. Finally, analyzing indicators of profitability, *grah poljak* production is profitable: EUR 100 of the production costs generates EUR 266 of profit or EUR 100 of generated revenues makes EUR 73 of profit. These figures are lower when it comes to conventional production of beans, where EUR 100 of the production costs generates EUR 187 of profit or EUR 100 of generated revenues makes EUR 65 of profit.

Previous comparative economic analysis indicates undoubted profitability and economic efficiency of production of *grah poljak* and achieving better economic performance compared to conventional cultivation. Production of *grah poljak* is more than profitable and main barrier to this production may be some other elements of the entire production cycle. This primarily refers to the sale and complete implementation of other marketing elements (distribution and promotion).

CONCLUSIONS

Typical agricultural and food products diversify the rural economy, contributing to the reduction of unemployment of rural population and provide greater employment opportunities for vulnerable categories of the rural population - women and youth. Typical agricultural and food products provide a developmental perspective to small farms to better valorize their potential. BiH agriculture is characterized by small sized farms. Limited resource farms work in mode of semi-natural production and they are characterized by small, unstable and insufficiently diversified surplus of products. These farms are insufficiently competitive, in terms of liberalization of domestic market many of them will not survive. These farms must find a (new) strategy for their survival and development, which includes diversification of revenues and activities, products and services of higher added value, including the typical products. In BiH there are number of agricultural and food products that can be characterized as typical products. Among the three products Slow Food recognized as typical in BiH, this paper analyses *grah poljak* from Petrovo polje - the municipality of Trebinje, which has the Ark of Taste status. Valorization and protection of such products is not only an act of legal recognition, nor the economic development of the product. Product protection is an extremely interesting model of development as it provides a range of development opportunities to the region from which product comes such as giving the new socio-cultural values to traditional and forgotten knowledge and skills, then the more measurable economic indicators such as increased employment, higher revenues and improved access to markets. Based on data collected from producers in the field, it is found that the profitability of production of *grah poljak* as a typical product, is much higher (profit of EUR 8,917 per ha) compared to conventional cultivation of beans (profit of EUR 3,329 per ha). Therefore, production of *grah poljak* is more than profitable and main barrier to this production may be some other elements of the entire production cycle. This primarily refers to the sale and complete implementation of other marketing elements (distribution and promotion).

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EFFECT OF CUT, STAGE OF GROWTH AND CULTIVAR ON ACID DETERGENT LIGNIN, PERMANGANATE LIGNIN AND KLASON LIGNIN IN RED CLOVER (*Trifolium pratense L.)*

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ABSTRACT: With advancing growth and maturity, forage cells insert a non-carbohydrate material, known as lignin, into the primary and secondary walls. Lignin can be thought of as the primary skeleton of the plant cell. It is important from a nutritional perspective because it is a non-digestible substance and its presence will inhibit the availability of the cellulose and hemicellulose portions of the forage. The experiment was designed as three factorial trials by randomized block system in three replicates. Three stages of growth of red clover (T*rifolium Pratense* L.) *cv* K-27 and K-39 were examined in the second and third cut. Three methods- acid detergent lignin (ADL), permanganate lignin (Per L) and klason lignin (KL) for determining lignin concentration of red clover were compared. Each of these methods gave different lignin values for the same type of forage sample. For all samples KL (Klason Lignin) values were higher ($P< 0.01$) than ADL (Acid Detergent Lignin) and Per L (Permanganate lignin) values. The KL residue concentrations were on the order of 1.60 to 2.20 times greater than the ADL and Per L residues. All three methods reflected maturity trends, although the magnitude of the change was different.
Key words: *red clover, klason lignin, permanganate lignin, cut, cultivar*

INTRODUCTION

Forages play an important role in providing nutrients for ruminants. The efficiency with which the potential energy of forages is utilized depends upon the type and maturity of the forage. Red clover (*Trifolium pratense* L.) is an important forage legume widely cultivated in most temperate regions around the world. In Serbia, red clover is one of the most important leguminous plants grown for forage production. Its success is based on the following characteristics: high seeding vigor, rapid growth, tolerance to acid and humid conditions, nitrogen fixing ability and high nutritive value for ruminants (Leto et al., 2004). The nutritive value of red clover decreases during plant development (Wilman and Altimimi, 1984). The increased content of cell wall constituents, especially lignin, is mainly responsible for this decrease.

Lignin plays a vital role in plant growth and development by improving water conduction through xylem tracheary elements, enhancing the strength of fibrous tissues, and limiting the spread of pathogens in plant tissues (Boerjan et al., 2003). Lignin restricts the degradation of structural polysaccharides by hydrolytic enzymes, thereby limiting the bioconversion of forages and fibrous crops into animal products or into liquid fuels and other industrial products. Lignified dietary fiber also plays an important role in maintaining gastrointestinal function and health in humans (Ferguson et al., 2001).

The various methods of lignin determination often give quite different estimates of lignin concentration (Fukushima and Hatfield, 2001, Fukushima and Hatfield, 2004). One major class of methods for lignin determination utilizes strong mineral acids to hydrolyze the other cell-wall components, leaving lignin as a residue to be measured gravimetrically. The other major class of methods employs oxidizing agents to remove the lignin selectively. In the second class of methods, lignin is estimated either by loss in mass of the sample or through a photometric assay for lignin oxidation products. The acid detergent lignin (ADL) procedure

of Van Soest et al. (1991) is the most commonly employed by animal scientists and agronomists for analysis of forages. There are both hydrolytic (H_2SO_4) and oxidative ($KMnO_4$) versions of the ADL method, the sulfuric acid variant of ADL is the most popular.
Our objective of this study was to compare the accuracy of the acid detergent lignin, permanganate lignin and klason lignin methods for measuring lignin concentration in red clover by employing an independent analytical method.

MATERIAL AND METHODS

The experiment was designed as three factorial trials, by randomized block system in three replicates. Three stages of growth of red clover (*Trifolium pratense* L.) *cv* K-27 and K-39 were examined in the second and third cut. Samples were hand cut with scissors at 5 cm height. The first stage was cut after 22 days of vegetation, at full boot stage, another one after 29 days of vegetation (around 40% flowering), and a third one in full flowering after 36 days of vegetation. Dry matter was determined by drying out samples at 65° C and grinding and sieving them to 1 mm particle size.
The acid detergent lignin (ADL) was determined as the lignin insoluble in 72% (w/w) sulfuric acid, applying the method of Van Soest and Robertson (1980). Briefly, ADL was isolated by filling the crucible containing the ADF with 12 M sulfuric acid and allowing it to drain from the crucible. Additional acid was added periodically over the 3 h room-temperature hydrolysis. The acid insoluble residue was collected by filtration and extensively washed with hot water. A final acetone rinse was used prior to drying the sample overnight at 100^o C. The ADL content was determined as the difference in weight of the residue before and after ashing at 450^o C.
The permanganate lignin (Per L) was determined as the residue remaining after oxidation with potassium permanganate by the method of Van Soest and Wine (1968). Briefly, Per L was isolated by filling the crucible containing the ADF with saturated potassium permanganate and buffer solution, 2:1, v/v. Additional this solution was added periodically over the 90 min. at 20-25^{o}C. After that, by filling crucible not more than half-full with demineralizing solution. The insoluble residue was collected by filtration and extensively washed with 80 % ethanol and acetone. The Per L content was determined as the difference in weight of the residue before and after ashing at 450^{o}C.
The Klason lignin (KL) was determined as the residue remaining after total hydrolysis of the cell wall polysaccharides by the method of Theander and Westerlund (1986). In brief, 0.1 g of samples were treated with α- amylase (Sigma, A 3403) and amyloglucosidase to remove starch, and solubilized wall polymers were recovered by precipitation in 80% (v/v) ethanol. Starch-free, alcohol-insoluble residues were solubilized in 1.5 ml of 12 M sulfuric acid at 30^o C for 60 min. The samples were than diluted with 43.5 ml of water to 0.4 M sulfuric acid prior to secondary hydrolysis in an autoclave (120^o C) for 60 min. The nonhydrolysed residue was collected by filtration through a coarse-porosity Gooch crucible, extensively washed with hot water and dried overnight at 100^o C. KL was determined as the difference in weight of the residue before and after ashing at 450^o C for 6 h.
Data were processed by the analysis of variance in a randomized block design. Effects were considered different based on significant ($P<0.01$) F-ratio. The significance of differences between arithmetic means was tested by LSD test.

RESULTS AND DISCUSSION

As forages mature there is generally a decline in digestibility of the fiber fraction that is associated with an increase in lignin. People have tried to use lignin concentration as a means of predicting cell wall and whole plant digestibility. One of the problems when trying to compare published data from a range of plants is that the type of lignin assay method used may vary.

The concentration of acid detergent lignin of red clover harvested at three different development stages in the second and third cut are presented in Table 1. All three main effects (cut, stage of growth and cultivar) were significant (P< 0.01) in these trials.
The cut x stage of growth interaction was also significant (P< 0.01). The results of this investigation indicated that ADL content was increased with advancing maturity in both cuts, and the mean values were from 38.50 to 57.17 g kg^{-1} of DM in the second and from 26.36 to 52.60 g kg^{-1} of DM in the third cut. The higher content of ADL was found in the second cut than in the third at all three stages of growth.
The cut x cultivar interaction was significant (P< 0.01), and there is evidence for both cuts that K-27 had considerably lower ADL content. In the second cut K-39 contained more ADL than K-27 by 4%, and in the third cut by 12.8%.
K-39 contained more ADL than K-27 at all sampling dates (P< 0.01). Mean values obtained in the present investigation were from 31.49 to 51.68 g kg^{-1} of DM in K-27, and from 33.37 to 58.09 g kg^{-1} of DM in K-39.
With growth and development of K-27 in the second cut ADL concentration increased after the first stage of growth by 19%, and after the second stage of growth by 23.5%. In the third cut, ADL content in this cultivar of red clover was from 24.77 to 47.16 g kg^{-1} of DM (P< 0.01). The content of ADL in K-39 was higher than in K-27 at all stages of plant development, except at the second development stage in the third cut.

Table 1. Content of Acid Detergent Lignin in red clover (*Trifolium pratense* L.), (g kg^{-1} of DM)

		A_1				A_2			
		B_1	**B_2**	**B_3**	$\bar{x}$ (AC)	**B_1**	**B_2**	**B_3**	$\bar{x}$ (AC)
Acid detergent lignin - ADL	**C_1**	38.22^{c}	45.49^{b}	56.19^{a}	**46.63^{b}**	24.77^{c}	36.32^{b}	47.16^{a}	**36.08^{b}**
	C_2	38.78^{c}	48.66^{b}	58.14^{a}	**48.53^{a}**	27.96^{c}	36.09^{b}	58.05^{a}	**40.70^{a}**
	$\bar{x}$ (AB)	**38.50^{c}**	**47.08^{b}**	**57.17^{a}**		**26.36^{c}**	**36.20^{b}**	**52.60^{a}**	
	$\bar{x}$ (A)		**47.58^{a}**				**38.39^{b}**		
	$\bar{x}$ (BC)	**31.49^{c}**	**40.90^{b}**		**51.68^{a}**	**33.37^{c}**	**42.38^{b}**		**58.09^{a}**
	$\bar{x}$ (B)		**32.43^{c}**		**41.64^{b}**			**54.88^{a}**	
	$\bar{x}$ (C)		**41.36^{b}**				**44.61^{a}**		

A_1 – second cut, A_2 – third cut; B_1 – first stage, B_2 – second stage, B_3 – third stage of growth; C_1 – cv K-27, C_2 – cv K-39. Different letters denote significantly different means (P< 0.01)

The another method of determining lignin content - permanganate lignin resulted in different amounts of residue, with Per L values were consistently higher than ADL values for all forage samples (Table 2). Lignin content in red clover differed significantly between cuts, stages of growth and cultivars (P< 0.01).
Permanganate lignin is based upon the ability to oxidize the lignin from a plant cell wall without affecting the cell wall carbohydrates. This method was developed originally for woody species in which the cell wall carbohydrate is predominately cellulose. In forages the cell wall contains significant amounts of other polysaccharides that are quite susceptible to oxidation by permanganate. For non-woody species this procedure tends to give much more variable results depending upon the cell wall make-up (Fukushima and Hatfield, 2002).
During maturation Per L increased from 47.43 to 77.70 g kg^{-1} of DM in the second cut, and from 34.35 to 71.32 g kg^{-1} of DM in the third cut. The highest increasing of Per L in red clover was after the first development stage in the second cut, and after the second development stage in the third cut. The cut x stage of growth interaction was significant (P< 0.01).
Cut x cultivar interaction was nonsignificant for concentration of permanganate lignin (Table 2).
There was an interaction (P< 0.01) between stage of growth and cultivar of red clover. Concentration of Per L increased with plant maturation from 42.93 to 72.24 g kg^{-1} of DM in K-27, and from 38.84 to 76.79 g kg^{-1} of DM in K-39. Despite the content of ADL in investigated

cultivars of red clover, K-27 contained more Per L than K-39 at all sampling dates, except at the third stage of development.
Concentration of permanganate lignin also increased as the plant matured in both investigated cultivars of red clover. In the second cut, K-27 contained higher concentration of Per L than K- 39 at the second and third development stages, whereas, values at the first stage of development were similar. In the third cut, K-27 contained higher concentration of Per L than K-39 at the first and second development stages, whereas much higher content of Per L was recorded in K-39 at the third stage of plant development.

Table 2. Content of Permanganate Lignin in red clover (*Trifolium pratense* L.), (g kg^{-1} of DM)

		A_1				A_2			
		B_1	**B_2**	**B_3**	$\bar{x}$ (AC)	**B_1**	**B_2**	**B_3**	$\bar{x}$ (AC)
Permanganate lignin - PerL	**C_1**	47.39^{c}	63.46^{b}	79.45^{a}	**63.43^{ns}**	38.46^{c}	53.55^{b}	65.02^{a}	**52.35^{ns}**
	C_2	47.46^{c}	61.58^{b}	75.96^{a}	**61.66^{ns}**	30.21^{c}	42.39^{b}	77.62^{a}	**50.07^{ns}**
	$\bar{x}$ (AB)	**47.43^{c}**	**62.52^{b}**	**77.70^{a}**		**34.35^{c}**	**47.97^{b}**	**71.32^{a}**	
	$\bar{x}$ (A)		**62.55^{a}**				**51.21^{b}**		
	$\bar{x}$ (BC)	**42.93^{c}**	**58.51^{b}**		**72.24^{a}**	**38.84^{c}**	**51.98^{b}**		**76.79^{a}**
	$\bar{x}$ (B)		**40.89^{c}**		**55.25^{b}**			**74.51^{a}**	
	$\bar{x}$ (C)		**57.89^{a}**				**55.87^{b}**		

A_1 – second cut, A_2 – third cut; B_1 – first stage, B_2 – second stage, B_3 – third stage of growth; C_1 – cv K-27, C_2 – cv K-39. Different letters denote significantly different means (P< 0.01)

Klason lignin is one of the oldest procedure and is frequently used for forage samples. The ADL procedure is basically the same except for the hot acid detergent treatment to insure the removal of protein and non-cellulosic polysaccharides before acid solubilization of cellulose (Fukushima and Hatfield, 2002).
Overall, as red clover matured, klason lignin increased (P< 0.01), with differences between cuts, stages of growth and cultivars.
The differences between stages of growth were less in the second cut than in the third cut (P< 0.01). The mean values were from 75.67 to 103.21 g kg^{-1} of DM in the second cut, and from 52.89 to 92.42 g kg^{-1} of DM in the third cut. Cut x cultivar interaction was also nonsignificant for concentration of klason lignin (Table 3). The two cultivars of red clover contained different amounts of klason lignin, with K-27 had consistently higher (P< 0.01) content than K-39.

Table 3. Content of Klason Lignin in red clover (*Trifolium pratense* L.), (g kg^{-1} of DM)

		A_1				A_2			
		B_1	**B_2**	**B_3**	$\bar{x}$ (AC)	**B_1**	**B_2**	**B_3**	$\bar{x}$ (AC)
Klason Lignin - KL	**C_1**	77.63^{c}	92.91^{b}	105.58^{a}	**92.04^{ns}**	51.78^{c}	80.49^{b}	92.02^{a}	**74.76^{ns}**
	C_2	73.70^{c}	88.43^{b}	100.83^{a}	**87.65^{ns}**	53.99^{c}	70.97^{b}	92.82^{a}	**72.59^{ns}**
	$\bar{x}$ (AB)	**75.67^{c}**	**90.67^{b}**	**103.21^{a}**		**52.89^{c}**	**75.73^{b}**	**92.42^{a}**	
	$\bar{x}$ (A)		**89.85^{a}**				**73.68^{b}**		
	$\bar{x}$ (BC)	**64.70^{c}**	**86.70^{b}**		**98.80^{a}**	**63.85^{c}**	**79.70^{b}**		**96.83^{a}**
	$\bar{x}$ (B)		**64.28^{c}**		**83.20^{b}**			**97.82^{a}**	
	$\bar{x}$ (C)		**83.40^{a}**				**80.12^{b}**		

A_1 – second cut, A_2 – third cut; B_1 – first stage, B_2 – second stage, B_3 – third stage of growth; C_1 – cv K-27, C_2 – cv K-39. Different letters denote significantly different means (P< 0.01)

In agreement with results of previous research (Jung *et al*., 1997) klason lignin concentrations were higher than corresponding ADL and Per L measurements for all samples in the present study**.**

It is generally considered that the major limitation of the KL method for forage samples is the inclusion of protein in the insoluble residue, resulting in artificially high lignin values (Van Soest *et al*., 1991). Cetyl trimethyl ammonium bromide (CTAB) is included in the acid detergent solution specifically for protein removal in the ADL method (Fukushima *et al*., 1991) whereas the KL method does not contain a protein removal step. Hatfield and Fukushima (2005) found large discrepancies between KL and ADL residues, particularly for grass forages. Their results indicated that ADL treatment of samples solubilized some of the lignin. This solubilization appeared to be extensive in grass samples. They concluded that major differences between concentrations of KL-generated residues and ADL residues were due to the loss of lignin as a result of ADL treatment rather than a large incorporation of proteins into the KL residue.
An advantage of the KL procedure is that it can be easily adapted to cell-wall hydrolysis schemes to obtain values for total carbohydrates, total uronic acid, and neutral sugar composition of forage samples, as well as estimating lignin content. The question remains, however, as to how accurately it estimates forage lignin content.
The present investigation has revealed that Klason lignin residues in red clover, especially at late stages of maturity, are very high. Lignin measured as the weight loss upon oxidation with permanganate was smaller than determined with sulfuric acid, agreeing with Hatfield *et al*. (1994.) The same authors reported that ADL values were 76% of those determined by the Per L method.

CONCLUSIONS

On the basis of the findings of this research, it is concluded that:
The Klason lignin method yielded greater estimates of lignin concentration in forages than did the sulfuric acid version of the acid detergent lignin and permanganate lignin method. A small part of this difference can be attributed to nitrogenous compound. The difference between KL and ADL values were of too great a magnitude to be accounted for simply as residual protein condensed in the residues.
The mean values of lignin content were greater in the second cut than in the third cut for all three methods.
The lignin content increased from first to third development stage in the second and the third cut in both investigated cultivars of red clover.
In both cuts, *cv* K-27 had lower ADL content than *cv* K-39 at all three stages of maturity.
cv K-27 had higher Per L and KL content than *cv* K-39 in the second cut.

ACKNOWLEDGEMENTS

Research was financed by the Ministry of Education and Science, Republic of Serbia TR 31057.

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EFFECT OF DIETARY FAT LEVEL ON BODY DIMENSIONS AND WEIGHT GAIN OF CARP

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ABSTRACT: The aim of this study was to investigate the impact of different level of fat in diets on body dimensions and weight gain of carp fry. Fish diets contained 38% protein and 8, 12 or 16% of fat. The experiment was carried out in triplicates, (3 tanks per treatment, 9 tanks in total) for 90 days at the Laboratory for Fish Nutrition, at the University of Belgrade, Faculty of Agriculture. Every tank has a usable volume of 120 gallons of water and water flow of 0.34 $Lmin^{-1}$. After adjusting fish to laboratory conditions, every tank was stocked with 29 fish, average weight 15.4 g.
Based on data of body length and height of fish, obtained in 30-to daily intervals, statistical analysis showed that there were differences among fish from different treatments. Fish fed diets with 8% fat achieved 46.8% higher weight gain than fish fed with food containing 12% fat and 65.4% higher weight gain than fish fed with feed containing 16% fat. Additionally, fish fed with less fat in diet had a more elongated body form and less pronounced body height as well as lower values for fish condition. Based on obtained results, it can be concluded that the diet with lower level of fat is justifiable to use, both from an economic and consumer perspective.
Key words: *carp fry, fat level, growth rate*

INTRODUCTION

The most commonly used fish feed in semi-intensive carp production are cereals. They contain a high percentage of carbohydrates and due to this is a primary source of energy for cultured fish (Sargent et al., 2002). When using grains as supplemental feed, the amount consumed per kilogram of growth rate usually ranges from 3.15 to 3.77, but is usually much more. This kind of feed in semi-intensive carp production results in increased body fat. Supplementary maize diet leads to carp body fat of about 13.26%. Body fat of fish fed supplementary wheat decreases down to approximately 11.22%, while the best results are attained by using triticale diet, producing only about 9.72% body fat (Vacha et al., 2007).
Fat in feed contribute to the better utilization of proteins from the meal, preventing its use as a source of energy (Skalli et al, 2004). Within certain limits, increasing levels of lipids in the diet, results in improved feed efficiency (Watanabe et al., 1979, Johnsen et al., 1993, Peres et al., 1999), providing biologically useful energy and thus sparing proteins. However, fish are able to utilize lipids in food to some extent, above which it may cause reduced growth, lowered food consumption (Ellis and Reigh, 1991) and formation of body fat. When feeding carp with a higher content of fat in diet, the final product is of poor quality, since the its level significantly affects the texture and consistency of fish meat (Huda, 2009).
In recent years, in order to boost production and improve meat quality of carp in South East Europe (Serbia, Bosnia, Romania, Bulgaria), cereals are more or less replaced by concentrated feed. By using supplementary concentrated feed better production results, higher feed efficiency and improved quality of end product, fish meat, are attained (Markovic, 2010). Concentrated feeds generally meet the nutritional needs of fish in terms of proteins, amino acids, vitamins and minerals, especially in periods of natural food deficiency. Nevertheless, the use of concentrated feed implies the necessity of larger financial resources for their purchase.

There is a variety of concentrated feed on the market composed from components of different origin, and various levels of protein and fat. In order to achieve maximum profitability in production, due to high cost of individual components (Aas et al., 2009), and achieve better production characteristics, it is important to know the nutritional needs of fish. By determining the nutritional requirements for fish, optimal production results are achieved with minimum investment.

The fat content of supplemental feed, except having direct effect on weight gain and utilization of feed has a relatively high impact on the characteristics of body dimensions: fork length and body weight (Kocour et al., 2007). Considering the market demand and consumer behavior, especially in Eastern European countries, which favor individuals of greater body size, the objective of this study was to investigate the effect of fat levels in concentrated feed on the weight gain and body dimensions of carp fry.

MATERIALS AND METHODS

The experiment was carried out at the Laboratory of Fish Nutrition, University of Belgrade, Faculty of Agriculture for 90 days. Three different types of concentrated feed were used (Table 1) with the same content of total proteins of 38% and 8, 12 and 16% of fat (Table 2), with 3 repetitions.

Table 1. Ingredients of experimental diets (%)

	Feed Am	**Feed Bm**	**Feed Cm**
Fish meal	18	18	18
Soy bean	22	30	30
Soy meal	16	11	11
Maize	7	6,4	3,4
Wheat	15,8	10,9	9,9
Yeast	15	15	15
Different row materials of plant origin	3	3	3
Oil	0	2. 5	6.5
Methionine	0.2	0.2	0.2
Lysine	0.4	0.4	0.4
$CaCO_3$	1	1	1
Monocalcium – phosphate	0.5	0.5	0.5
NaCl	0.1	0.1	0.1
Mineral and vitamin premix	1	1	1

Table 2. Chemical composition of the experimental diets

	Feed Am	**Feed Bm**	**Feed Cm**
Dry matter (DM) gkg^{-1}	944	966	970
In DM (g)			
Proteins	379.9	381.3	377.8
Fat	80.3	119.4	157.9
Ash	88	81	82
Cellulose	29.9	28,8	29,9
[1]Nitrogen-Free Extract (NFE), g	42.2	39	35.2
[2]Row energy (MJ/kg)	19.4	20.5	21.2
[3]P/E	19.6	18.6	17.8

1Nitrogen-Free Extract (NFE), g = 100 – proteins (g) – fat (g) – ash (g) – cellulose (g)
*[2]Row energy = protein (g) * 23,6 + fat (g) * 39,5 + NFE (g) *17,3*
[3]P/E = Protein-energy ratio (g proteins $(kJ)^{-1}$ row energy

The experiment was performed in 9 tanks with usable volume of 120 gallons of water and continuous flow of dechlorinated tap water of 0.34 $Lmin^{-1}$. After adjusting fish to laboratory conditions, every tank was stocked with 29 fish, average weight 15.4 g. Fish were fed daily using automatic belt feeders (AGK Kronawitter GmbH) 3% of their body mass. Water

temperature, oxygen concentration and oxygen saturation were recorded at 10 min intervals using OxiGuard system.
In 30-day intervals body weight was measured using a digital balance a digital balance Radwag THB - 600, (accuracy 0.01 g). Fork length and height were measured using the ichthyometer.
Following equations were used for calculating:

Body weight gain: $BWG\ (\%) = (Wt - Wo) / Wo \times 100$,
Wt – final body weight (g) and Wo – initial body weight

Condition factor: $CF = (W / L3) \times 100$,
W - body weight (g) and L – fork length (cm).

Length to height ratio of: $CB = L / H$
L - fork length (cm) and H - total fish height (cm).

Data were examined by one-way analysis of variance with feed as the factor and two-way analysis of variance with factors feed and period. Differences between treatments were tested using the Tukey test.

RESULTS AND DISCUSSION

At the beginning of the experiment, fish had homogenous body dimensions (CV<30%). Average fish body dimensions had no statistically significant differences ($p>0.05$). During the experiment, no significant differences were found for water temperature ($p=0.805$) and oxygen saturation ($p=0.275$). Due to this, it can be presumed that all the differences obtained during the experiment where the effect of different diets.
After the 90 - day experiment, weight gain ranged from 213.9% in fish that received the feed with 16% fat, to 352.2% in fish from the treatment with 8% fat in the diet. Fish fed with 8% fat, achieved 46.8% and 65.4% higher weight gain than fish from the other two treatments. Statistically significant differences were found in the weight gain of fish between treatments with 8% and 12% and 16% fat in the diet ($p <0.001$). Similar results are recorded by Yilmaz et al. (2005), who achieved better results in weight gain of carp fry by applying lower fat diet. By using mixtures with 7% fat, they attained 40% higher weight gain than when using the mixture containing 11% fat, and by 52.1% using 15.4% fat in the mixture. However, research by Manjappa et al. (2002) show that the increase of fat content in the diet has a positive effect on the weight gain up to some extent. Further increase will lead to lower weight gain. However, they pointed out that fish fed feed containing 11.4% fat, achieved 30.6% better weight gain than fish fed with 6.8% fat and 9.4% higher final weight than carp fry fed diet with 9.1% fat. Further increase of fat in the diet from 11.4% to 13% led to a decrease of weight gain by 14% in the four-month study.
Condition factor ranged on average from 1.72 for fish from the treatment with 8% fat in the mixture to over 1.75 for fish in treatment with 12% fat and 1.77 for fish fed with 16% fat. Tukey-HSD test confirmed that the obtained values of condition factor were significantly different between fish fed with 8 and 12% fat ($p = 0.021$), and between fish receiving 8 and 16% fat in the diet ($p = 0.001$). Similar results were obtained by Skoric et al. (2012) for condition factor in carp from the semi-intensive production system, but with no statistically significant difference.
The average fork length was significantly different between the three treatments ($p <0.001$), while the average height differed in fish from treatment with 8% body fat compared to fish with 12% ($p = 0.003$) and fish fed 16% fat ($p =0.001$) in the diet. Conversely, no statistically significant difference was found in the length to height ratio of fish from different treatments.

CONCLUSIONS

Based on the results, it can be concluded that it is more reasonable, both from economic and consumer perspective, to use lower level of fat (around 8%) in feed for carp. As the final goal, fish produced except larger final weight, have a slightly elongated body form and much better meat quality.

ACKNOWLEDGEMENTS

The present study was supported by the Ministry of Education and Science, through the project Improvement of production capacities of carp (*Cyprinus carpio)* through nutrition and selective breeding programs (TR 31075).

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THE IMPORTANCE OF THE TRANSITION PERIOD TO ORGANIC MILK PRODUCTION

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ABSTRACT: Organic farming primarily aims to produce food with high nutritive value, to maintain or increase soil fertility, prevent and combat soil erosion, biodiversity conservation and to protect natural resources from pollution. In order to base organic production it is necessary to provide the spatial isolation of land parcels and farms of the possible sources of pollution (pesticides, waste water, pollen of genetically modified crops), the plot of land on which the content of harmful substances does not exceed the prescribed maximum level, and proper irrigation water quality (first and second category) and air.
There are many studies that show the health benefits of organic milk compared to milk from conventional production. Organic milk has higher content of polyunsaturated fatty acids and omega-3 fatty acids, more vitamin A, C and α-tocopherol, it does not contain residues of antibiotics, pesticides or other chemicals. Organic milk is healthier because the cows are kept free (in the barn and in the discharge) and they are long time on pasture and without stress.
Although Serbia has an interest for organic milk production, for the health and economic reasons, there is still no market certified milk or other dairy products.
In order to make transition to organic production it must happen through a transition period or the period of conversion to switch from conventional to organic farming. Methods of organic farming in livestock production have to be applicable from the beginning of the conversion period, which begins after the conclusion of the contract on the exercise of control in organic production between the manufacturer and the authorized control organization.
The paper presents a method of organic milk production in period of conversion on a farm in Futog Agricultural High School, where special attention is paid to the following procedures: agro-ecological conditions, the number of animals per hectare, race, diet and posture, health and reproduction of animals, and parallel production (conventional and organic farms at the same farm), cadastral parcel numbers, history of land, fertilization, seed sourcing, analysis of water, controlling weeds, pests and diseases and the effect of biological protection and record-keeping. Detailed implementation of measures of organic plant and livestock production on farms in the period of conversion, which are consistent with the Law on organic farming and regulations on professional standards, are make to avoid the mistakes in next year and ensure the transition to organic milk production.
Key words*: transition period, conversion period, farm, organic milk*

INTRODUCTION

Worldwide demand for organic products is growing rapidly from year to year, particularly in Europe (Popović Vranješ et al. 2011) and also the number of farms switching from conventional to organic production. Most European countries recognize organic farming as a sustainable, eco-friendly production that does not pollute the environment and people as consumers of these products. Philosophy of organic food production is based on and reflects several principles, such as: biodiversity, ecological balance, sustainability, natural soil fertilization and natural pest management. Organic agriculture is possible on the entire farm, part of farm or family farms (Eco-farms) with compatible crop and livestock production, energy balanced, and environmentally and horticulturally developed space.
Procedure of involving producers in organic production is initiated by the manufacturer who submits application to authorized control organization. Based on the application, the

manufacturer and the authorized control organization shall conclude a contract on the performance of control and certification. The transitional period is a time that is necessary to establish a system of farm management, build soil fertility and its biological activity, develop sustainable agro ecosystem and reduces the use of chemicals or their residues produced by using conventional methods of agriculture. Depending on the results of the required chemical analyzes (including the control of land use in the previous three years and production program for the transitional period), transitional period lasting 2 to 3 years will be defined.
In order to establish patterns of organic milk production, it must meet precisely defined conditions, such as isolation of land parcels, livestock farms and processing facilities from potential sources of pollution, water quality, coordinated development of crop and livestock production and the ability of producers for the organic agriculture with the obligation of constant knowledge innovation. The organic production is needed to ensure that the breeding of animals provide organic fertilizer, without which there is no organic production, i.e, the sustainability of land (Lazarević, 2008). Dairy cattle must have access to pasture for at least 120 days a year. All animals must have access to pasture, whenever weather conditions permits, since the land on which pastures are based should not apply manure or composted manure, which is not entirely biological decomposed (www.albertamilk.com). All animals older than 6 months must have access to pasture. Antibiotics, preparations based on synthetic organisms and GMO products are not allowed in food preparation or during the treatment of animals. The use of antibiotics is permitted, except, only in special situations, where its use must be documented, and it is allowed to use up to two times a year. Dairy animals that require more than 2 treatments per year must undergo 12 month conversion period, and those with chronic problems need to be extracted from the herd. Organic milk compared to conventional, has a better fatty acid composition and contains more polyunsaturated fatty acids and omega-3-fatty acids (Ellis et al., 2006; Baltušnikiene et al., 2008; Prandin et al., 2009).

MATERIAL AND METHODS

The aim of this paper was the research concentrated on creating conditions for organic milk production in frame of Agricultural School from Futog which entered the process of introducing organic crop and livestock production by Organic Control System Ltd. from Subotica. During the inspection, checking of documents and parcels registered for the first year of conversion to a total area of 14.80 ha was performed. On that parcels period of two years transition period began from the date of signing the contract. Sampling and analysis of soil on the content of hazardous substances on the reported parcels were performed. Part of livestock production which will be reported for organic milk production is dairy cattle, so a physical and chemical analysis of raw milk from the conversion period was performed as well as the contents of somatic cells and the total number of bacteria by standard analytical methods. Milk samples from transition period was analyzed monthly, from Holstein and Simmental cows, and the results are presented as mean values.

RESULTS AND DISCUSSION

In the transition period, conventional dairy farms establishe the conditions for the production on the principles of organic production. These activities include; assessment of requirements, selection of animals, provision of appropriate housing, care, nutrition and health monitoring, and production of quality milk and establish records of all activities on the farm management database, according to the requirements of certification bodies, which controls and monitors the production in the transition period. It is necessary to make a plan (sketch) of the parcels that go into a period of transition, as to make it easier to plan activities related to the plan of planting and rotating crops and forage, and ease of recording completed activities predicted by certification bodies (Figure 1).

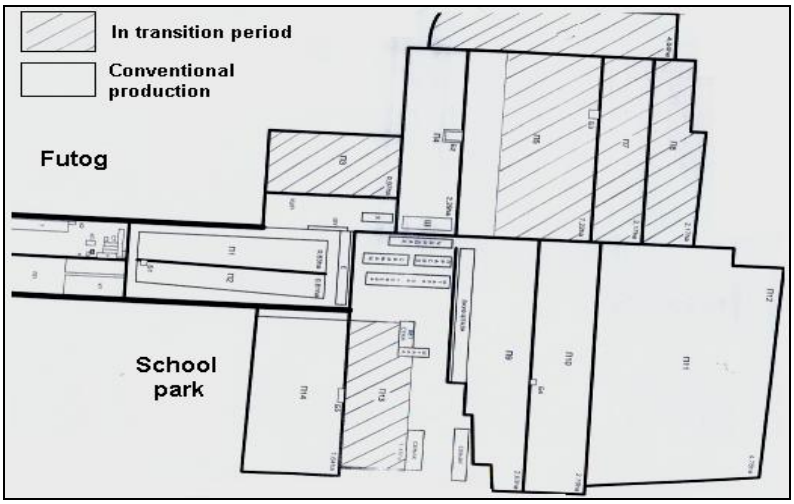


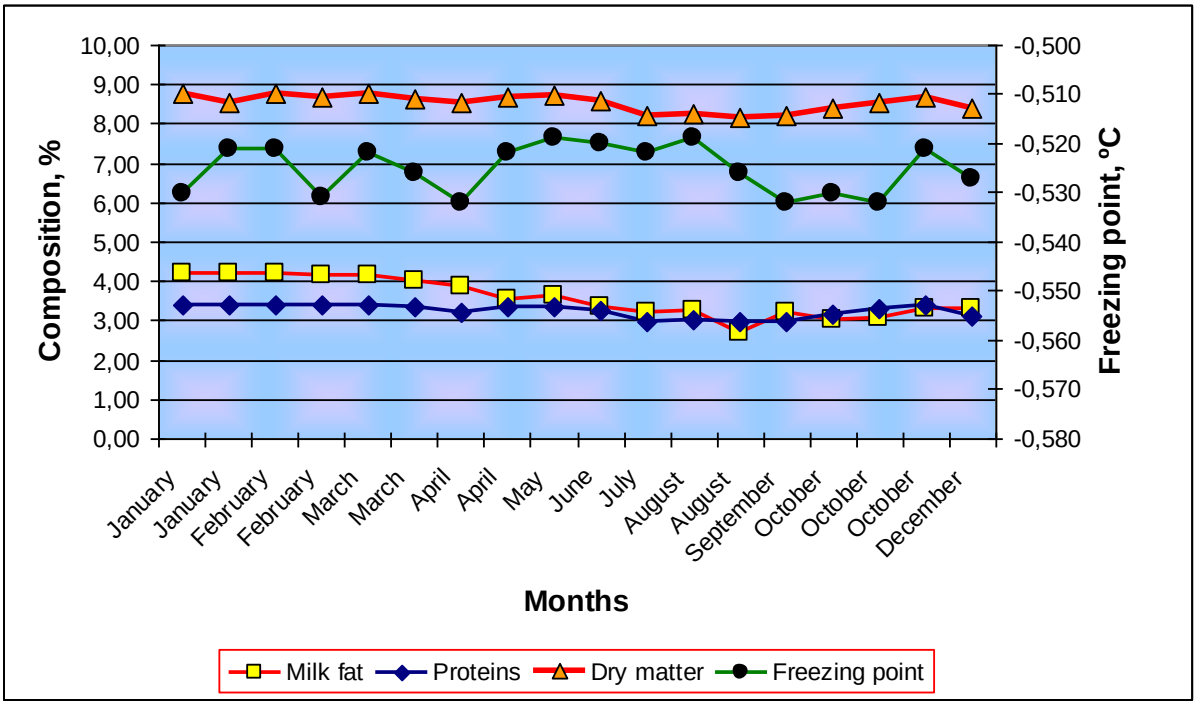
Figure 1. Sketch of parcels involved in the transition period

Milk from organic production is obtained from animals to which shall be provided growing conditions that allow expression of the natural functions and behaviors, because they form part of the agro ecological systems. In organic production, to farm animals must be given the appropriate conditions for breeding, counting on their wellfare and their health in accordance with the type and breed of animals. Cows are kept in open stables with enough space to move and to rest with protection from atmospheric conditions and the stress generated due to the occurrence of a large number of animals in small spaces as well as ammonia, Figure 2. Milking takes place in a modern milking parlor, which along with proper hygiene of milking and udder give milk with a small number of somatic cells and the total number of microorganisms. Milking parlor must be located and constructed so as to ensure satisfactory hygienic conditions during milking and easy to maintain hygiene (Krajinović et al. 2011). Today there are a lot of milk producers who are seriously considering about processing the whole or part of their production of milk into cheese within their household as a model of mini dairy. Given that investments in the small size household dairies are low, and products achieves a high price on the market, such forms of production are high profitable. The second production model could be a medium sized dairy plant (craft dairy manufacturers), which potentially involves higher volume of production that is sold on market, Figure 3. All ingredients used in the production and processing of organic milk and milk products should be organically produced, except for additives and auxiliaries allowed in the production of organic products (Miličić et al. 2010).

Figure 2. Stable for the free keeping system

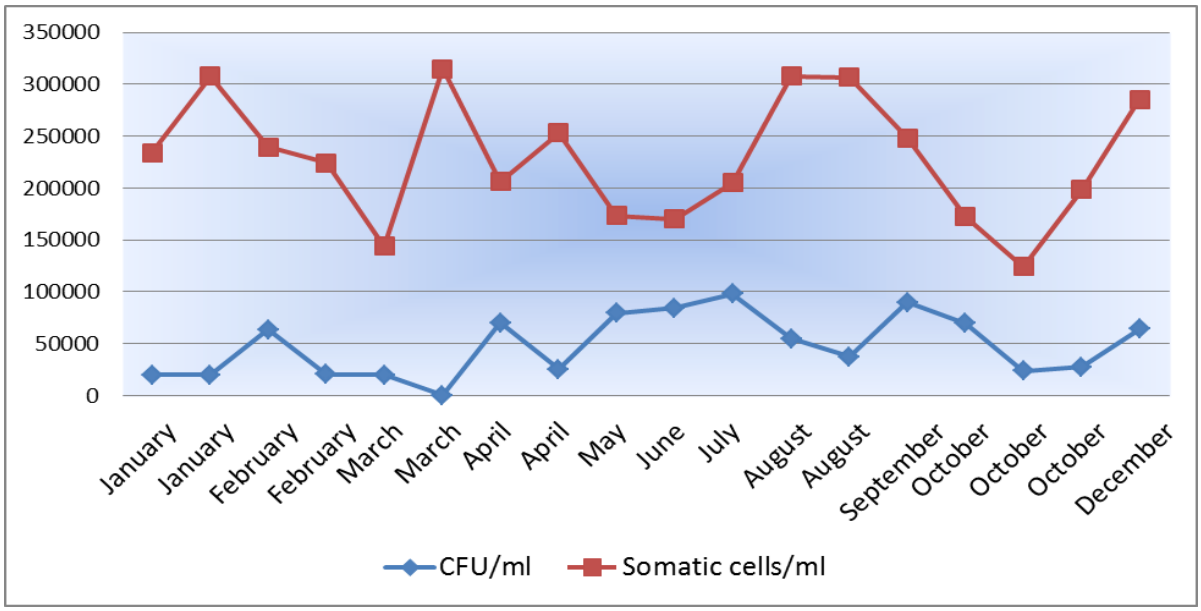
Figure 3. Dairy for the processing of organic milk

Animal health is a very important item to which the milk producers in the transition period must pay attention. Uterine infection and the occurrence of mastitis are two problems that organic farmers are often meeting. The appearance of mastitis is very important because it reduces the production, quality and income from milk and is a major problem, depending on the types of microorganisms that cause it. With good milk production managment, milking and hygiene can be reduced or completely eliminated the risk of mastitis (Tigner 2007). Therefore, intensive monitoring of udder health for early detection of new cases of mastitis, as well as proactive adaptation of mastitis control management strategies are essential for farmers who are engaged in milk production during transition period. If a contagious mastitis is present in the herd that are planned for conversion to organic production and processing of milk, all efforts should be focused on the identification, treatment, and/or excretion of infected animals before the conversion period (Tikofsky, 2010). It is necessary to continuously control the composition of milk in order to identify errors in the nutrition, hygiene and animal health and to eliminate faults, Graph 1.



Graph 1. Physical and chemical parameters of milk quality from the transition period

The analysis of milk from the period of transition was determined that the milk fat content ranged from 3.08 to 4.20%, protein content from 2.95 to 3.40% and dry matter without fat from 8.16 to 8.78%. Freezing point ranged from 0.519 to 0.532 ºC below zero. The quality of milk was constant throughout the year with certain variations caused by stage of lactation, season and other factors important for milk production. This is particularly marked for freezing point of milk which is in a large dependence on diet, season, water intake, cows breed, time of day (morning / evening milk), etc. Control of somatic cells contents and the total number of microorganisms is extremely important from the aspect of mastitis control and health status of dairy cattle, and hygienic conditions during production and milking. The total number of microorganisms in milk from the period of transition was below 100 000/ml, and somatic cell below 400 000/ml according to which milk was of the extra class quality, Graph 2.



Graph 2. Changes in the number of somatic cells and the total number of microorganisms in milk during the year

The average content of microorganisms in milk amounted to 48894.33 cfu/ml, and somatic cell 180,444.4/ml. Based on the results from graph 1 and 2 it can be established that the content of certain components of milk (milk fat, protein, dry matter) had a stable trend during the year, thanks to the proper nutrition of cows. The contents of somatic cells and the total number of bacteria showed a monthly variation in which the obtained values are not exceeded the maximum allowed. Bennedsgaard et al. (2003) found that cow health and milk quality of organic farms are better than conventional, and is directly proportional to the length of time that is spent in an organic farm production system, i.e the longer milk cows in the organic farming system are, the better is quality of milk. Milk from organic livestock production does not contain antibiotics and other harmful residues harmful to the health of consumers.

For the certification process and the transition of conventional to organic production, particularly important are professional control of authorized certification body, where reports should be prepared on: the plan and rotation of crops, number of hectares that go under transition (number of cadastral parcels), the history of each parcel during 3 years (previous culture, fertilizing, controlling weeds, pests and diseases), fertilization and types of fertilizers, the quality of water for irrigation, livestock, which resides on the farm (number and races of animals, housing and nutrition. Given that the fodder are supplied from own fields, it is necessary to analyze soil to determine the content of harmful substances that would eventually passed into the food or milk. Soil analysis results obtained, showed that the registered lands quality meets the requirements for growing the crops for dairy cows.

Table 5. The total content of hazardous and adverse substances in the soil from the transition period intended for the production of animal feed

Lab. No.	Cu	Zn	Co	Mn	As	Pb	Cd	Ni	Cr
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
1	27,5	63,5	9,9	496,6	8,4	19,1	nd	28,2	32,7
2	41,6	78,8	11,6	530,2	8,2	13	nd	33,7	37,6
3	34,7	67,3	9,5	497,4	8,3	11,6	nd	27,5	40
4	24,9	57,7	9,2	454,7	8,6	17,6	nd	25,4	27,8
5	36,6	65,3	10,9	522,8	9,8	17,1	nd	32	36,2
6	27,7	63,9	10,9	513,5	10,2	16,5	nd	30,7	34,3
MAC*	100	300			25	100	3	50	100

MAC*= Maximum allowable concentration. The maximum amount allowed by the Regulations on permitted amounts of hazardous and adverse substances in soil and water for irrigation and methods of their analysis (RS Official Gazette 23/1994).

CONCLUSIONS

Testing the quality of milk shows that there was no mastitis in the herd, because the determined number of somatic cells was at the level of completely healthy animals. The total number of bacteria in milk was on first class quality level (<100 000 cfu / l). The results of physico-chemical properties of soil have shown that land from the aspect of organic production is not contaminated with heavy matals and other chemicals. The animals were housed in a buildings that fits the requirements for organic livestock production. Animals were kept in accordance with the principles that allow welfare of animals (they have access to food and water at all times) and other conditions which ensures good health and maximum production of milk by the quality and quantity.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 31095 financed by the Ministry of Science and Technological Development, Republic of Serbia.

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NITROGEN FERTILIZATION AND MYCOTOXIN ACCUMULATION INFLUENCE UPON PROTEIN AND AMINO ACID CONTENT OF CORN GRAINS

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ABSTRACT*:* Because they make up a large part of diets, corn grains cannot be considered only as a source of energy, as they provide significant amounts of protein as well. It is also recognized that cereal grains have a low protein concentration and that protein quality is limited by deficiencies in some essential amino acids, mainly lysine. For a balanced nutrition is important not only the amount of amino acids taken over, but the ratio between them, because the disproportion in feed of amino acids composition leads to a complex disorder of protein metabolism. The content of amino acids in plant biomass is affected by N nutrition. In this paper the protein content, and amino acid composition of corn grains was determined. Corn (Zea mays L.) was grown in a series of field plots which received various rata of applied nitrogen: 50 kgN·ha^{-1}, 100 kgN·ha^{-1}, 150 kgN·ha^{-1} and 200 kgN·ha^{-1}. Amino acid composition, after subjecting samples to conditions which would hydrolyze protein, was determined by ion chromatographic method. The analyses were made on samples with and without mycotoxins accumulation. The amino acid content varied significantly with protein content of the samples. Nitrogen rate that have a significant effect on the maximal accumulation of valine, isoleucine, leucine, phenylalanine, histidine, and alanine was 150 kgN·ha-1. Higher nitrogen application rates alter the amino acid balance thereby reducing the nutritional value. Mycotoxins accumulation took place in samples fertilized with 200 kgN ·ha^{-1}, which had lower amino acid content that samples without mycotoxins.
Key words: *corn, nitrogen fertilization, amino acid, protein*

INTRODUCTION

Corn or maize is one of the most popular cereals in the world. Because they make up a large part of diets, corn grains cannot be considered only as a source of energy, as they provide significant amounts of protein as well. The corn kernel is composed of approximately 72% starch, 10% protein, 5% oil, 2% sugar, and 1% ash with the remainder being water (Perry, 1988). It is also recognized that cereal grains have a low protein concentration and that protein quality is limited by deficiencies in some essential amino acids, mainly lysine.

Fertilization has a significant influence upon protein yield on surface unit and its quality. Fertilizers trough sortiments, doses and application periods determines protein and amino acids quantity in plants. Although the genetic potential is decisive for the protein content in plants, protein quantity and ratio of certain amino acids may be influenced by the fertilization (Radulov et.al., 2010).

Nitrogen (N) nutrition is one of the major factors limiting growth and production of crop plants (Losak et.al., 2010). The content of amino acids, important N-containing compounds, in plant biomass is also affected by nitrogen nutrition (Neuberg et al. 2010, Pavlík et al. 2010). Nitrate and ammonium are the major sources of N for plants. Ammonium-supplemented plants often show a higher concentration of amino acids than nitrate-supplemented plants, while higher N supply also causes an overall increase in amino acid content (Atanasova 2008).

Increasing nitrogen supply to corn generally resulted in increased grain and protein yields and increased grain protein concentration (Blumenthal et al., 2008). It is apparent that the amount of fertilizer nitrogen required to maximize grain yields is not the same as the amount that will produce maximum grain protein concentrations (Sander et al., 1987). MacGregor et al. (1961) found that amino acid concentrations of grain did not increase uniformly to nitrogen

fertilizer application, and that the concentrations of lysine, methionine, and phenylalanine did not increase. Rendig and Broadbent (1979) found that nitrogen fertilizer application decreased the concentrations of tryptophan, lysine, glycine, arginine, and threonine in protein, while concentrations of alanine, phenylalanine, tyrosine, glutamic acid, and leucine were increased.

Mullins *et al*., (1998) reported that, field experimentation involving different rates of nitrogen fertilizer increased forage protein content in corn. Where nitrogen levels are limiting, photosynthesis is not fully used in the synthesis of organic nitrogen compounds and sugars are accumulated. Heier et.al. (2005) showed that mycotoxin contamination in maize was influenced by N-fertilization rather than by the treatments. In 2001, the DON content was significantly increased due to the conventional N-supply. Immoderate N-fertilization however, can increase mycotoxin levels significantly even under conditions unfavourable for **Fusarium** spp.

Several factors influence toxin production in the field and in culture. One of these factors is nitrogen source. Stimulation of toxin production by amino acids is interesting for two reasons. First, water stress causes an increase in free amino acids. Of the amino acids that accumulate, proline usually accumulates to the greatest extent and is the amino acid that most consistently increases in several plant species, including corn. Second, proline has a variety of roles in microorganisms other than as a protein constituent. In non-halotolerant gram-positive bacteria, accumulation of specific free amino acids is important in cell osmoregulation, and in several species of bacteria proline appears to be the main amino acid involved in osmoregulation. Proline has also been reported to stimulate microbial growth, Payne and Hagler (1983) showed that proline with other amino acids and nitrogen sources are known to stimulate aflatoxin.

The aim of this work is to establish nitrogen fertilization and mycotoxin accumulation influence upon corn grains protein content and amino acids composition.

MATERIAL AND METHOD

A series of corn samples, hybrid Lovrin 400, fertilized with different fertilizers was studied, for ten years period, in pedoclimatical conditions from USAMVB Timisoara. The researches have been made on the cambic chernozem with middle texture, with following properties: total density ranged between 2.43 g|cmc and 2.58 g|cmc, total porosity has medium values, excepting the soil surface were total porosity has highest value: 47%; soil reaction is weakly acid, pH=6.18; humus content of soil is ranged between 3.28 si 2.10%, nitrogen index is ranged between 2.04 and 3.08 %; phosphorus soil content is low – 13.0 ppm, and potassium content of soil is medium– 184 ppm; the value of cationic exchange capacity of soil is 30.35 me/100g.

The samples were obtained from field plots receiving mineral fertilization: different doses of phosphorus and potassium P_0K_0 – control (0 kg P_2O_5 and $K_2O \cdot ha^{-1}$); $P_{50}K_{50}$ (50 kg P_2O_5 and $K_2O \cdot ha^{-1}$); $P_{100}K_{100}$ (100 kg P_2O_5 and $K_2O \cdot ha^{-1}$); $P_{150}K_{150}$ (150 kg P_2O_5 and $K_2O \cdot ha^{-1}$) on pre-fertilized plots with nitrogen N_0 – control (0 kg $N \cdot ha^{-1}$); N_{50} (50 kg $N \cdot ha^{-1}$); N_{100} (100 kg $N \cdot ha^{-1}$); N_{150} (150 kg $N \cdot ha^{-1}$); N_{200} (200 kg $N \cdot ha^{-1}$).

The corn samples were finely ground and dried for 24 hours at 60°C . Raw protein content from corn grain was determined by Kjeldahl method, as Kjeldahl nitrogen multiplied with 6.25.

The amino acids were assayed using ion-exchange chromatography after hydrolizing with 6 M HCl for 24h at 110°C. Methionine and cystine were analyzed by using formic acid protection prior to acid hydrolysis.

The chromatographic conditions are: DIONEX ICS-3000 Amino Analyzer, AMINOPAC PA10 Analytical Column (2x250 mm, P/N 055406), AMINOPAC PA10 Analytical Guard Column (2x50 mm, P/N 055407), Mobile phase: E1: water, E2: NaOH 250 mM, E3: NaAc 1 M, Reference electrode: pH/Ag/AgCl, Flush volume: 250 μL, Flow rate: 0,25 mL/min, Column temperature 30^0C. The minimum detection levels of standard was 5 ng/L for each of the amino acids and have been established based on signal to noise ratios of 3:1. The linear

dynamic range of the detector response was checked. The average correlation coefficient was between 0,9884-0,994.

The values obtained are expressed as per cent of a given amino acid in the whole grain. All values are expressed on the basis of the moisture free samples. The original samples contained 13 to 14% moisture. Statistical analyses were performed using SAS computer program.

RESULTS AND DISCUSSIONS

Nitrogen is of special importance because plants need it in rather large amounts, it is fairly expensive to supply, and it is easily lost from the soil. A major factor in successful farming is the farmer's ability to manage nitrogen efficiently. An abundant supply of the essential nitrogen compounds is required in each plant cell for a good rate of reproduction, growth, and respiration. The proper functioning of nitrogen in plant nutrition requires that the other essential elements, particularly phosphorus and potassium.

Protein content of corn grains, after nitrogen fertilization on plots pre-fertilized with phosphorus and potassium is presented in table 1.

Table 1. Nitrogen fertilization influence on raw protein content of corn grains (%)

Fertilization variant	kg ha^{-1} P_2O_5 and K_2O			
	0	50	100	150
	% raw protein			
0 kg ha^{-1} N	8,25	8,31	8,33	8,40
50 kg ha^{-1} N	8,57	8,67	8,71	8,78
100 kg ha^{-1} N	8,98	9,02*	9,00	9,05*
150 kg ha^{-1} N	9,68**	9,79**	9,24**	9,25**
200 kg ha^{-1} N	9,26**	9,40**	9,80**	9,78**

** * Significant at 0,1 percent level, * Significant at 1 percent level*

The highest protein content was determined in variants with high nitrogen fertilizers doses on all plots pre-fertilized with phosphorus and potassium. The raw protein content of corn grains rises as the nitrogen dose rise. Experiments done by Blumenthal et al., 2008, showed that increasing nitrogen supply to corn generally resulted in increased grain and protein yields and increased grain protein concentration. The corn samples from plots fertilized with 200 kg·ha^{-1} N and with no or low (50 kg·ha^{-1}) phosphorus and potassium fertilization presented mycotoxins accumulation. In this plots, raw protein content was lower than one in plots fertilized with 150 kg·ha^{-1}N. Although phosphorus and potassium does not influence raw protein content in such a large extend like nitrogen, they sustain nitrogen effect and attenuates negative effects of high nitrogen doses upon protein quantity and quality. The highest protein content of corn grains was determined after application of 200 kg·ha^{-1}N on plots pre-fertilized with 100 kg·ha^{-1}P_2O_5 and K_2O (9,80%) and 150 kg·ha^{-1}P_2O_5 and K_2O (9,78%). Corn samples from this variants didn`t presented mycotoxin accumulation. Similar results were presented by Heier et al. (2005) they showed that N-fertilization influenced mycotoxin contamination in maize.

Many researches showed that amount of fertilizer nitrogen required to maximize grain yields is not the same as the amount that will produce maximum grain protein concentrations. Nitrogen fertilization influence upon amino acids content of corn grains was determined only in plots pre-fertilized with 50 kg·ha^{-1}P_2O_5 and K_2O, which presented mycotoxin contamination, and 150 kg·ha^{-1}P_2O_5 and K_2O, without mycotoxin contamination.

Table 2. Amino acid content of corn grains fertilized after nitrogen fertilization on plots pre-fertilized with 50 kg·ha-1phosphorus and potassium (%)

AA	kg ha^{-1} N				
	0	50	100	150	200
Alanine	0,45	0.59	0,62	0,70	0,63
Arginine	0,22	0.36	0,48	0,45	0,46
Aspartic acid	0,41	0.53	0,60	0,69	0,68
Cystine	0,08	0,18	0,22	0,26	0,27
Glutamic acid	1,05	1,20	1,40	1,53	1,50
Glycine	0,13	0,21	0,24	0,39	0,39
Histidine	0,10	0,17	0,22	0,31	0,32
Isoleucine	0,14	0,26	0,28	0,32	0,33
Leucine	0,55	0,80	0,91	1,03	0,98
Lysine	-	-	0,15	0,22	0,20
Methionine	-	0,10	0,11	0,13	0,14
Phenylalanine	0,20	0,35	0,42	0,40	0,38
Proline	0,35	0,67	0,71	0,80	0,76
Serine	0,18	0,30	0,33	0,36	0,37
Threonine	0,20	0,30	0,29	0,33	0,34
Tryptophan	-	0,016	0,028	0,031	0,028
Tyrosine	0,18	0,34	0,33	0,40	0,41
Valine	0,22	0,43	0,48	0,49	0,51
Total	4,46	6,80	7,81	8,84	8,69

On plots pre-fertilized with 50 kg·$ha^{-1}$$P_2O_5$ and K_2O, total amino acid content rises with increasing dose of nitrogen fertilizer. Exception makes application of 200 kg·ha^{-1}N, were the content (8,69%) was lower than in variant fertilized with 150 kg·$ha^{-1}$$P_2O_5$ and K_2O (8,84%). Corn sample from plot fertilized with 200 kg·ha^{-1}N presented mycotoxin contamination. Among the amino acids the highest quantity was determined in glutamic acid in all fertilization variants (table 2). Experiments made by Seebauer et al. (2004) with increasing dose of nitrogen fertilizer (0 and 168 kg N/ha) had a variable effect on individual amino acid levels in young maize cobs. However increasing N concentrations were associated with decrease in crude protein of lysine, methionine, cystine, threonine, tryptophan and, generally, with increase in isoleucine, leucine, phenylalanine and glutamate (Losak et.al., 2010).

Table 3. Amino acid content of corn grains fertilized after nitrogen fertilization on plots pre-fertilized with 150 kg·ha^{-1} phosphorus and potassium (%)

AA	kg ha^{-1} N				
	0	50	100	150	200
Alanine	0,60	0,69	0,65	0,75	0,70
Arginine	0,42	0,46	0,45	0,50	0,53
Aspartic acid	0,61	0,63	0,64	0,75	0,75
Cystine	0,14	0,28	0,19	0,32	0,34
Glutamic acid	1,15	1,30	1,40	1,59	1,60
Glycine	0,23	0,24	0,28	0,45	0,46
Histidine	0,15	0,17	0,20	0,37	0,39
Isoleucine	0,25	0,26	0,30	0,38	0,40
Leucine	0,70	0,80	0,95	1,10	1,05
Lysine	-	-	0,10	0,28	0,24
Methionine	-	0,15	0,11	0,20	0,21
Phenylalanine	0,30	0,35	0,38	0,46	0,46
Proline	0,55	0,67	0,70	0,86	0,82
Serine	0,28	0,30	0,29	0,43	0,44
Threonine	0,30	0,30	0,30	0,40	0,41
Tryptophan	-	0,026	0,031	0,038	0,035
Tyrosine	0,28	0,34	0,36	0,45	0,48
Valine	0,42	0,43	0,45	0,55	0,58
Total	6,38	7,40	7,78	9,87	9,89

A possible reason for the lower values of total amino acids content in plots pre-fertilized with only 50 kg·ha^{-1}P_2O_5 and K_2O could be nitrogen fertilization applied in an exaggerated report to other nutrients, such a phosphorus and potassium.
In the variant were nitrogen wasn`t applied lysine, methyonine and tryptophan wasn`t determined (Table 2, and table 3). In variant were 50 kg·ha^{-1}N was applied only lysine was absent.
In plots pre-fertilized with 100 kg·ha^{-1} P_2O_5 and K_2O the amino acid total content was higher than in plot pre-fertilized with 150 kg·ha^{-1} P_2O_5 and K_2O. The total highest values was determined in plot fertilized with 200 kg·ha^{-1} N (9,89%). Application of highest nitrogen dose lead to a decrease of alanine, leucine, lysine, proline and tryptophan content. Losak et al. in their experiment made in 2008 determined that the highest N dose (treatment N2) significantly decreased the content of threonine, valine, iosleucine and leucine compared with the lower N dose. Kniep and Mason (1991) found nitrogen application increased grain yield, protein concentration and percent lysine of sample, but decreased percent lysine of protein. The predominant amino acids are aspartic acid and glutamic acid. Rendig and Broadbent (1979) found that nitrogen fertilizer application decreased the concentrations of tryptophan, lysine, glycine, arginine, and threonine in protein, while concentrations of alanine, phenylalanine, tyrosine, glutamic acid, and leucine were increased.

CONCLUSIONS

Application of highest nitrogen dose, on plots without phosphorus and potassium fertilization, determined mycotoxin accumulation in corn grains. In the variant were nitrogen wasn`t applied lysine, methyonine and tryptophan wasn`t determined. In variant were 50 kg·ha^{-1}N was applied only lysine was absent. Fertilization with highest nitrogen dose (200 kg·ha^{-1}N) lead to a decrease of alanine, leucine, lysine, proline and tryptophan content. Samples with mycotoxin accumulation had lower amino acid content that samples without mycotoxins.

AKNOWLEDGEMENT

This study was achieved from research project funds: *Systems to reduce mycotoxins contamination of cereals and medicinal plants in order to preservation native species and traditional products in Romania - Serbia - Croatia area,* financed by SEE-ERA NET PLUS Program, Contract reference ERA_139/01, 2010-2012.

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IMPROVING THE EFFICIENCY OF THE WHEAT SUPPLY CHAIN

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ABSTRACT: Food production is the basis for country development. On the global market will always do well food producers whose costs are lower. Today, consumers demand of food requirements are increasing in quality, taste, healthy and cheaper price. The attention is also on environment of food production. The competition in market are focused on value for consumers where corporate various actors in food production. Suppliers, manufacturers, distributors, and retailers are all have various industry initiatives to gain competitive advantage. Whether these initiatives take the form of better service, lower prices, or some combination of both, they all share a common essence: integrating the supply chain. All of this has consequences for the way in which the various actors in the food chain cooperate. Investments must be taken place in the area of freezing foods, storage, packaging, fruit processing. It is also good to investing in new equipment and production lines for vegetable processing, greenhouse primary production and modern irrigation systems. However, the investments in our country are usually relatively small and may also stay relatively small in the future. This paper provides a general overview of the Serbian wheat supply chain, and the associated infrastructure and processes of the key elements of the export wheat supply chain. The export wheat supply chain producing Serbia is then summarized, including the participants in each segment of the chain. In this paper we give some basic guidelines for improving and increasing the efficiency of the supply chain for wheat with special emphasis on the qualitative and quantitative parameters of silos for wheat storing.
Key words: *supply chain, wheat, efficiency, improvement, silos*

INTRODUCTION

The literature in the supply chain management field defines a supply chain as a set of facilities, technologies, suppliers, customers, products, and methods of distribution (Arntzen et. al., 1995). The basis of supply chain management is logistics as opposed to accounting or strategy. The problem with the supply chain is essentially finding the optimal balance between cost and quality.
Logistics is the process of planning, implementing, and controlling the efficient, cost-effective flow and storage of raw materials, in-process inventory, finished goods, and related information from point-of-origin to point-of-consumption for the purpose of conforming to customer requirements (Lambert et al., 1998).

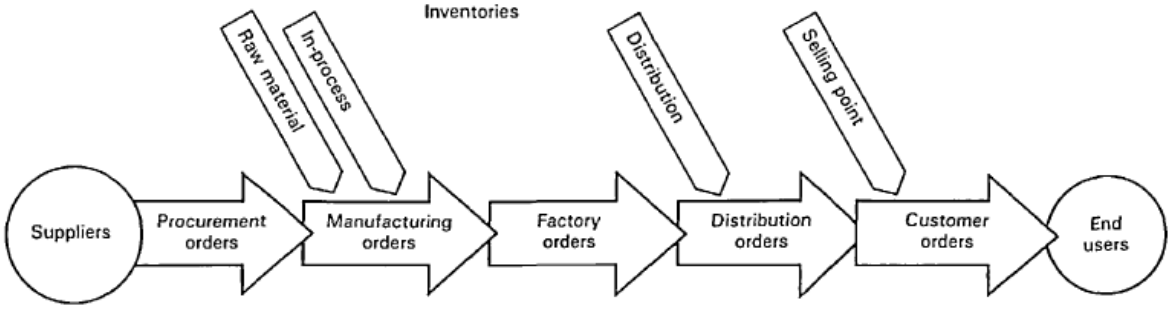

Figure 1. Supply chain management (Houlihan, 1985)

A basic barrier to the application of supply chain management is the traditional organization of most enterprises. Firms and supply chains are made up of separate production, distribution, and sales organizations often with conflicting objectives. To alleviate these conflicts, firms and managers must view their activities as a continuous flow of both products and information with the focus being to accelerate them. This focus on product and information flows is often depicted through the concept of a pipeline (Figure 2).

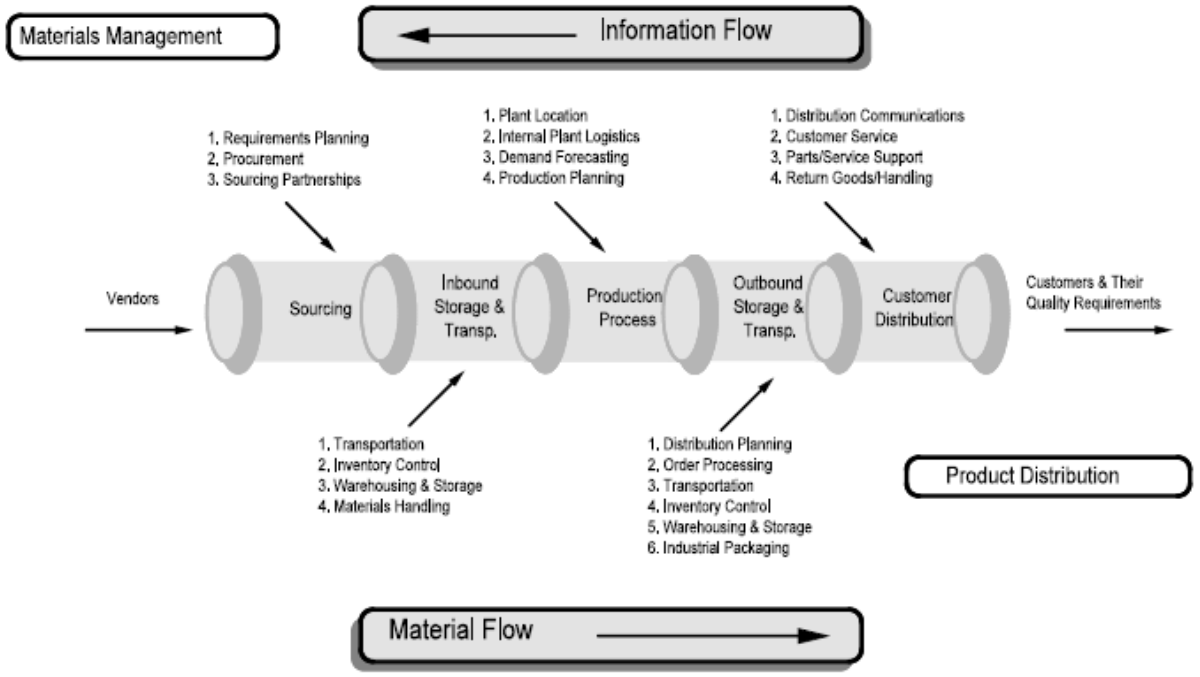

Figure 2. The logistics pipeline (Coyle et al., 1992)

WHEAT PRODUCTION IN TNE SERBIA

Serbia has very favourable climate conditions for agricultural production with 4.2 million ha of arable land (of the total of 5 million ha agricultural land) (Food Industry Study in Southeast Europe, 2010). Over the last few years, Serbia has established itself as a net exporter of cereals and edible oil. Agricultural products are mostly exported to CEFTA countries, EU and Russian Federation. However, most of the field crop production is with low yield, significantly below potential.

Flour production in the Serbia is currently running at over 500,000 tonnes per annum. The industry uses about 700,000 tonnes of wheat for this production, which represents only one third of the total wheat production of the country. This indicates the potential importance of the grey market in flour production. Official statistics are limited. Records about the production of bread and bakery products are also imprecise and the latest estimates say that the value of unregistered trade is about 10 billion Dinars. About 6500 companies are estimated to operate in this sector of which only 300 are registered. The Ministry of Trade has announced the possible introduction of excise stamps for flour in order to put an end to grey trade. Quality standards have been completely neglected in the supply chain of wheat, flour and bread production due to the current market conditions. This is why bread of poor quality is eaten in Serbia and the supply of special kinds of bread and other products made of flour is modest and expensive (Food Industry Study in Southeast Europe, 2010).

ELEMENTS OF THE WHEAT SUPPLY CHAIN

The wheat supply chain in Serbia are characterized by a lack of professional supervision in production process, incompetent market presence, and illegal sales. The supply chain is

comprised of producers, storage and processing facilities, industry, commodity market, wholesalers and retailers (Figure 3).

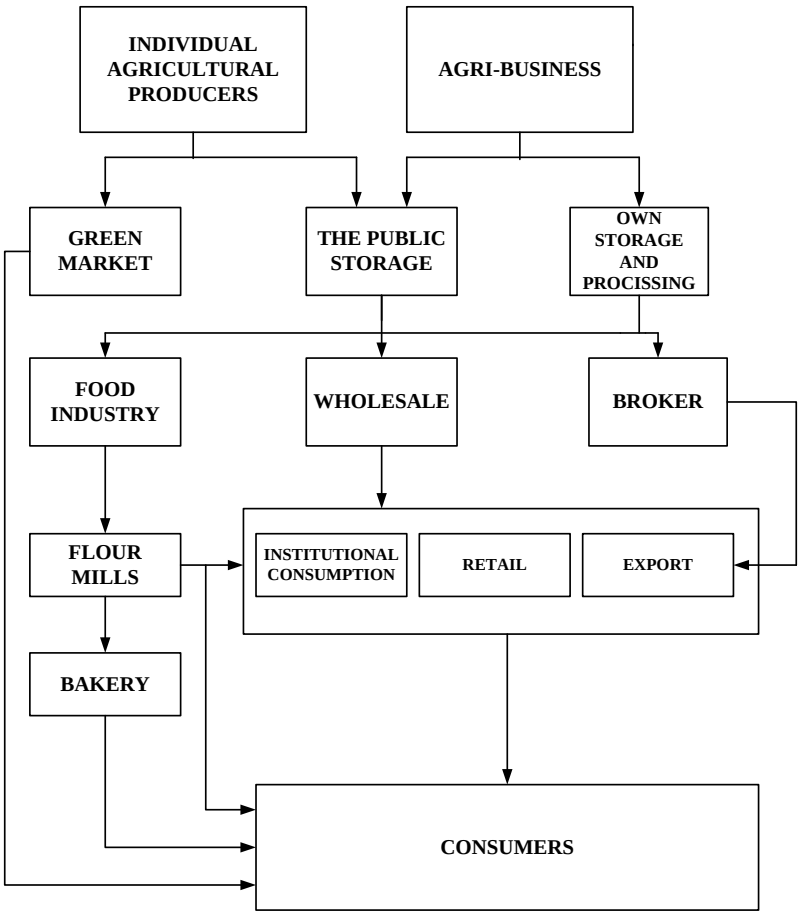

Figure 3. Wheat supply chain in Serbia

The structure of the wheat flour milling industry has changed in the last few decades. This industry segment is typical of the structural dynamics confronting other segments of the agricultural processing industry. Flour milling accounts for over 90 percent of domestic wheat processing use. The primary product is wheat flour for baking, while by-products are used for such things as livestock feed, pet food, and industrial applications.
Wheat processing capacity is around 1.2 million tonnes per year. There are enough storage and processing facilities, but they are mostly worn with low hygienic conditions.
There are three most important links in the wheat value chain: storage, flour millers, and bakers. Each of these links represents an important economic activity within the supply chain. Although heavily intertwined, each link competes in a unique economic environment. We considered in detail the storage technology.

STORAGE TECHNOLOGY

The role of storage is to preserve the quality of products received at the storage. Therefore the storage for wheat in the process of receiving goods in storage, preparation for storage, the storing and issuance of wheat from storage should comply with a set of activities presented in Figure 4. In addition to the above processes, storage should carry out the preparatory activities to enable the above processes be implemented in an optimal way (Vukicevic, 1995).

Quality preservation activities in the storage

In the order to preserve the quality, quantity and condition of stored wheat applied echnological operations: operations on elevators with or without fine cleaning, aeration, cooling, storage in an inert atmosphere and keeping with the addition of chemically inert dust.

In the storage for wheat in Serbia operations on elevators are dominant, and it could be said currently the only acivity that is widely used. This activities represent transfer process of stored wheat through a system of internal transport from one silo cell to another with or without grain leakage through the silo cleaning aspirater for fine grain cleaning. Plan for operations in elevator creates storage technologist on the basis of prevention planning for operations in elevator and intervention plan, made up according to the values determined critical indicators of stored wheat condition. This plan based on the guidelines of standardization and homogenization, too. As a rule, stored wheat, if there is no need for intervention operations in elevator, it is necessary and sufficient that 3 to 4 times a year pass through operations in the elevator.

In the operations in elevators reduces the wheat temperature, moisture balances, Aeris is, remove the foreign smells that may occur (the smell of stale). In order to achieve these effects completely, it is necessary to done that activities only when weather conditions are favorable for the performance of this technological measure, especially when the humidity is low and when there is no precipitation (Vukicevic, 1995).

CLASSIFICATION AND CHARACTERISTICS OF SILOS FOR WHEAT

Important element in the wheat supply chain are silo. Therefore, in terms of increasing efficiency of the supply chain we give a review basic types of silos, their characteristics and opportunities for process improvement of storing and other processes occurring in silo (Cooper et al., 1997).

In many silos in construction has been installed equipment to operate the silo and equipment for monitoring the condition of stored goods, but as a result of years of exploitation and technical advances come across a very bad picture of the condition of both pieces of equipment. Technically satisfactory those silos that are privately owned.

Generally, the problem is that capacities are not fully utilized, considered to be somewhere around 60% and that do not follow of possibilities in the range field, but it is processed by only the base product.

Cube-shaped silo may be constructed in a wide range of different cell number and total capacity. Size of the cubic cell is determined according to the needs of the customer or applied technology for processing, and their number by the total required capacity. According to the purpose and function of the cube-shaped silo can be constructed as: on farms silos, industrial silos, commercial loading silo, a silo complexes.

Silos on farms are less capacity appropriate to the quantities of wheat, which are produced and consumed on the farm. Equipped with the a admission line of road vehicles, equipment for cleaning, drying, filling and emptying. On the silos ground floor the optimal solution is to be installed mixers for the production of concentrated feed on the central part, from one side to form a storage for packaged components, on the other side of the pack, with a third party storage the finished product (concentrate in sacks). Triangular base of the pyramid-shaped silo allows approach from each of the three sides for loading or unloading packaged goods, so there is no crossing and mixing of material flows and the paths are shortest. Granular component (grains) are located in the cells above the silo mixers (their participation in the concentrate is about 60 - 70%), and in the processing are introduced simply by using gravity. In the basement under the silos can be three smaller rooms for administration, control samples, a workshop for minor repairs and spare parts for equipment. In the rest of the basement can be organized animal feed production, with use as raw grains from the silo. Classical solutions applied in the practice now have two to three times higher committed location, special facilities for the silo, blending, storage components, animal feed, administration, then the longer routes, equipment for connecting objects, long installation,

greater energy consumption, etc. In our case, all these functions are integrated into a single object on one basis, and investment savings are about 40% (Beamon, 1998).

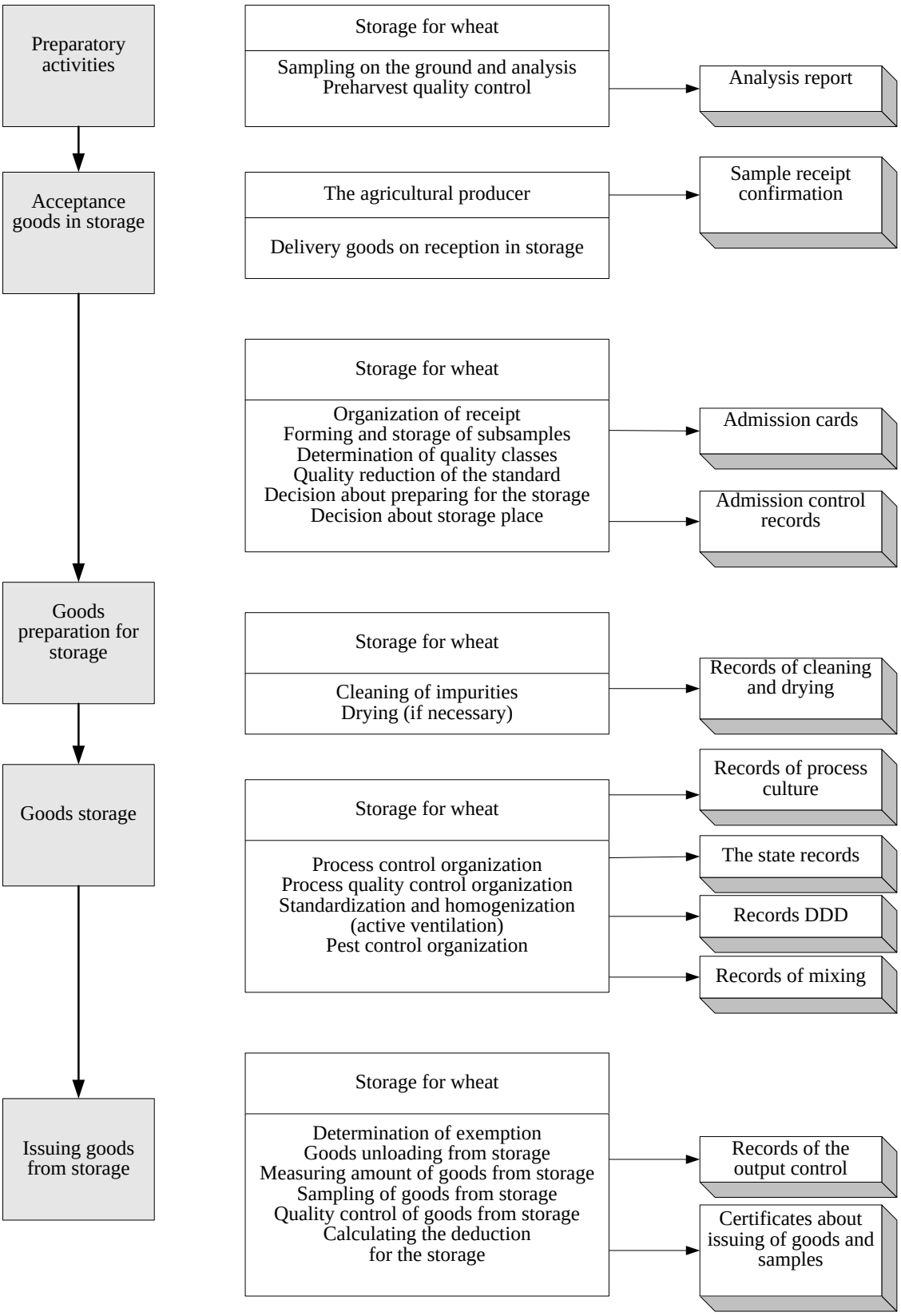

Figure 4. Flow chart of technological processes in storage for wheat (Mastilović et al., 2011)

Industrial silo are much larger capacity (10000-20000 tons) is required in a basic purpose. Can be constructed for receiving, drying, cleaning, storage and processing of wheat and other grains, corn, soybeans, etc. These silo have a larger capacity production lines, can have a railway terminal or more receiving lines. They can be built as single objects with processing facilities, such as mills for wheat, corn mills for human consumption, for the preparation of soybean plants, larger animal feed factories, etc. Then, they can be built near the existing silo and processing capacities, as an extension of grain storage in bulk, but should count on a considerable acreage under silo that are automatically granted with the silo construction. These surfaces can be used as a storage floor, as well as space for the installation of equipment for primary processing of grain, or installation of lines with several processing stages with a greater degree of product finalization, to the level of small packages based on resources from the primary production (wheat - flour - pasta, corn - flour - extruded products, soy - preparation - cattle feed), with additional programs such as sprouts for human consumption, etc. In the beer industry, for example, where silo can be constructed for barley malt, and that automatically gets a small space on the tight locations of breweries, several thousand square meters of storage floor, for example, crates for beer boxes and so on. One such silo could significantly reduce investment in a seed center and up to 40%, if in the cells were stored seed grain and in the area under silo can be located line for seed processing, packaging and storage until the time of distribution.
Reloading trade and port silo are generally large capacity silo (content of the goods) and large handling capacity of transport equipment. They are characterized by high frequency grain of various kinds. Therefore, it should be great to be able to accept all goods quantity as soon as possible carry out the same loading combinations: truck - silo - ship, truck - silo - railway wagon, railway wagon - silo - the ship and vice versa. So, they must have sufficient size and number of required terminals, but they are not equipped with the machines and dryers for cleaning, because the goods come dry and clean and a very short time remains for the silo. We particularly emphasize the importance of large storage floor at these sites that can often be more expensive than just buildings that are built on them, and storage facilities at ports are always in short supply, and storage services is very expensive.
Large silo complexes are largely with industrial character. They are built to the individual capacity of 20,000 tons and from a few of these silo can forming a complex on the principles of separation technologies that are not compatible, move closer to the rational connection of compatible technologies, rational transport organization, energy supply, installation, movement of workers and meeting their needs (Lambert et al., 2001).

CONCLUSIONS

The largest market for wheat from Serbia are the CEFTA countries. Serbia does not wheat quality for export. In the past four years, Serbia to the EU exported wheat for less than $ 40 million and wheat flour for about two million. Serbia is a price-competitive in the wheat production, primarily due to lower earnings of our farmers, on one hand, and lower land prices, on the other. However, wheat stocks are transferred from year to year and sown area decrease. The primary reason for this is - poor quality. Our wheat is most often classified as feed rather than food quality. The primary reason for this is very bad quality of our seeds. Seeds of wheat is expensive, which makes it in most cases, farmers do not buy, but they sow "from the attic". This seed can not achieve the required quality for export. Serbia has one seed house that has a monopoly on the market that behaves logical in a given situation: does not care about quality and price. So, as long as the market does not find a better and more productive wheat seeds, although Serbia will not significantly price-competitive export wheat in the EU. Serbia has the potential and all conditions to be a significant exporter of wheat (a large proportion of arable land in total land, favorable climate, small farmers' income expectations, sufficient storage space, etc.) and with the date of entry into the EU, be an important supplier of wheat in CEFTA countries and the EU. On the other hand, Serbia could easily become an occasional importer of wheat, if the manufacturer does not allow to have:
• Greater competition in the seed selection,

• Lower cost and higher seeds quality, which will allow greater use of the declared seed,
• Affordable control option for the application of mineral fertilizers,
• Merging the amount of subsidies to neighboring countries,
• Renewal and modernization of machinery and storage areas under favorable conditions.

To achieve this scenario, customs needed be abolished in whole or in less than 15% because high transport costs burdening the import (primarily due to the low value tonnes of wheat).

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III 46001 “Development and application of new and traditional technologies in the production of competitive food products with added value for european and world market - Create wealth from wealth of Serbia”, funded by Ministry of education and science, Republic of Serbia.

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NUTRITIONAL CHALLENGES AND AGRICULTURAL STRATEGIES IN NATIONAL NUTRITION POLICIES OF CENTRAL AND EASTERN EUROPEAN COUNTRIES

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ABSTRACT: Central and Eastern European countries experience significant socio-economic changes and are characterized by nutrition transition. They deal with persistent undernutrition and micronutrient deficiencies while being confronted with emerging consequences of overnutrition and diet related non-communicable diseases. Effective solutions will require investment in contextual drivers of diet, in particular in agriculture.
This study examines how national nutrition policies of Central and Eastern European countries published between 2004-2011, articulate agricultural measures to address nutritional challenges: undernutrition, micronutrient deficiencies, food safety and overnutrition using structured content analysis. Only 5 (24%) national documents (Bulgaria, Georgia, Moldova, Poland and Slovenia) out of 21 countries officially belonging to Central and Eastern European region were retrieved that met research criteria. Agricultural measures to tackle food security in vulnerable population groups mainly propose mechanisms to support agricultural production in rural settings and facilitated access (e.g. through subsidies) of healthy foods (e.g. fruits and vegetables) for vulnerable groups. Other strategies entailed education of producers and consumers about healthy food consumption, fortification of staples with micronutrients and establishment of food safety systems throughout whole food chain.
Strategies aimed at the agricultural sector for better diets in CEE region are interesting examples to generate lessons learned and provide information to further integrate agriculture and nutrition research for countries in nutrition transition.
Key words*: agriculture, nutrition, micronutrient deficiencies, food safety*

INTRODUCTION

Central and Eastern European countries have been experiencing vast political, economical and social changes in the last 10 years. They are however, still coping with persisting problems such as social inequalities, poverty and causally food insecurity. In many of these countries more than half of the population lives in rural areas and is poorer then urban citizens. They are predominantly engaged in agricultural activities as a mean of employment and income. Even though basic infrastructure services such as roads, electricity, clean water, education and health services are available, as a legacy from socialist system, there is a lack of integrated strategies to use the assets present to address numerous social problems (Mizik, 2010).
With regard to this, the nutrition status of the population in the region is one of the factors highly affected. Malnutrition is persistent amongst vulnerable groups- poor, mothers and children and elderly, and it is mainly characterized by nutritional deficiencies in iron, zinc or overall malnutrition due to poor diets (Rokx et al., 2002). Moreover, other nutritional problems such as overnutrition and obesity are prevalent and drive the development of diet related non-communicable diseases epidemic in the region. An estimated 80% of the adults are obese (Knai *et al*., 2007). The epidemic of non-communicable diseases contributes further to

an estimated 77% of the total loss in DALYs (%) and affects mainly poor and people in rural communities (Pomerleau *et al*., 2002).
Finally, food safety monitoring, that concerns quality of food available to the people and access to the foreign markets and international competitiveness, especially in EU, is poorly developed in the legal frameworks of countries in the region. In countries which joined the EU after 2004, the application for EU membership acted as a catalyst for adjustment of legal frameworks and implementation of food safety standards and guidelines. Similarly candidates countries for EU accession, such as Croatia, are passing through a similar adaptation process, while some other countries (Armenia, Georgia) that are coping with the poverty, experiencing difficulties in transition processes (Mizik, 2010).
The reality is that agriculture and nutrition (and health) are dealt with in separate contexts. A comprehensive approach at national level is seldom adopted. Food and nutrition which act as dominant cause of contemporary health conditions in various forms (malnutrition, deficiencies, overnutrition, food poisoning) is considered as an isolated issue under Ministries of Health or other, such as agriculture and social affairs. On the other side, the development in agriculture is usually observed through an increase in production, price trends, access to the markets and other economical indicators. Agricultural policies and strategies are designed under the supervision of Ministries of Agriculture and Economy which influence the trend of their development. In this context, nutrition remains idle in political and institutional frameworks (Fan & Brezska, 2012). So far, nutrition has not been widely used as an objective in development strategies considering both agriculture and health. There is a need for evidence based policy making and to formulate strategies for concerted action in nutrition. These strategies have to take into account activities such as the promotion of productivity growth of more nutritious foods; increase in demands for and access to nutritious foods along the entire food chain through consumer knowledge and awareness campaigns; breeding more nutritious varieties of staples that are consumed by the poor through the use of e.g. biofortification practices, or even the introduction of taxes on unhealthy foods and complementary subsidies on nutrient-rich foods.
Altogether, the nature of agricultural policies – essentially mostly perceived in terms of food production - equally needs to provide people with adequate quality and complete nutrition and provides livelihoods for the rural poor. The development of agricultural policies consequently has a significant public health impact. Despite this, however, broad societal and public health consequences of agricultural policies are usually neglected (Hoddinott J., 2012). To give consideration to such comprehensive approach, policy makers have to be informed on different aspects of implementation of nutrition strategies in public policies through information that includes data on nutritional patterns, micronutrient intake data etc. (Fan & Brezska, 2012).
This study analyses the nutritional policies of Central and Eastern Europe with the perspective of reviewing agriculture initiatives that are relevant for nutrition. It will examine how policies design agricultural strategies to address issues of undernutrition, overnutrition, nutrient deficiencies and food safety. It aspires to give an idea on current situation in terms of nutrition policy development in CEE countries and to point out alternative ways of thinking about plausible and clearly necessary segments in building new effective food and nutrition policies.

METHODS AND MATERIALS

Nutrition and diet related policy documents were retrieved by study group from Faculty of Bioscience Engineering, Gent University, using following inclusion criteria (i) officially approved policy of a WHO member state; (ii) publicly available document, published between 2004-2011 and (iii) written in English. Selection of the documents for this study was done from this collection, where we took all available documents for the countries that officially belong to Central and Eastern Europe group. The entire data set of policy documents was used for other research analysis by the study group, as well.

Information regarding the agricultural strategies was extracted manually from the text following a structured content analysis approach. In this analysis process, key issues regarding agricultural strategies were: food security, micronutrient deficiencies, food safety and overnutrition. The contextual of the policy documents was extracted for the various pieces of coded text to allow further consideration, interpretation and comparison. The extracted information was summarised and is presented in tables to facilitate comparisons and to allow a comprehensive evaluation.

RESULTS AND DISCUSSION

The Central and Eastern European sub-region consist of 21 countries out of which only 5 (24%) national documents (Bulgaria, Georgia, Moldova, Poland and Slovenia) were retained for review as they were the only available nutrition related policies officially approved, satisfying research criteria. Table 1 lists the documents used for analysis in the study.

Table 1. List of official national documents used for analysis

Country	Title	Publisher	Year
Bulgaria	Draft Food and Nutrition Action Plan	Council of Ministries	2005-2010
Georgia	"Food security, healthy eating and physical activity" national policy	Public Health Department of Georgia (Ministry of Labour, Health and Social Affairs)	2006-2010
Slovenia	The national programme of food and nutrition policy	Official gazette of Republic of Slovenia	2005-2010
Moldova	National Health Policy- Republic of Moldova	Ministry of Health	2007-2021
Poland	National prevention programme of overweight, obesity and non-communicable disease through diet and physical activity improvement	Ministry of Health, National Food and Nutrition Institute	2007-2016

Prior to presenting concrete results, it is key to point out that, even though a total number of endorsed policies are small, other countries in the region have been active in development of various strategies to address nutritional needs of their people and are at different stages of nutrition/food policy development. These activities could be seen through formation of capacity development network of regional professionals in CEE in 2006 suggested by UNU/SCN. This network have indentified major common nutritional challenges of all the countries in the region, which included: irregular meal patterns, low consumption of fruits and vegetables, milk and fish, low intake of micronutrients and high intake of fat, sugar and salt (Pavlovic *et al.,* 2009).

Countries such as Serbia, Hungary, Romania, Croatia, Albania etc. are facing various challenges in formulating policies. These problems are multifactorial e.g. limited interaction between ministries, governmental bodies and overall rigid political system which do not recognise the need for the policy, lack of coordinated nutritional activities i.e. monitoring systems. Furthermore, higher education and training on nutrition, which are prerequisites for the establishment of nutrition policy, are inadequate both for medical and agriculture professionals. Tools for building policies- food based dietary guidelines, recommended dietary allowances, food databases, are also randomly developed in those countries (Pavlovic *et al*., 2009).

The next section provides an overview of agricultural measure to address food security, micronutrient deficiencies, promotion of healthy foods and food safety in analysed CEE policies. Table 2 provides an overview of the main strategies for nutrition and agriculture in the analysed policies.

Table 2. Overview of agricultural strategies proposed in national nutrition and health policies in CEE region

	Country/ Strategies	**Bulgaria**	**Georgia**	**Slovenia**	**Poland**	**Moldova**
1	**Support agricultural productivity to ensure food security for vulnerable groups: children elderly poor disabled**	Develop mechanisms for production of food for elderly Economic and financial support for fruits and vegetable and fish production Create conditions for access to the market Milk and fruits for school children	Investment in agriculture to obtain food security in rural economy Subsidy program. for disadvantaged groups Programs of healthy foods in schools	Implement projects to facilitate access of poor to healthy foods Strengthening of self supply – anti-crisis	-	Support agriculture production and facilitate access of foods to vulnerable
2	**Measures to tackle problems of micronutrient deficiencies (MND)**	Fortification of staples with Fe, folic acid	Agriculture response to MND	Production of food for those with special needs	-	Apply new technology for fortification of staple food (Fe, I, folic acid)
3	**Promotion of food with health-benefit-production, processing, trade, distribution**	Improve awareness of food operators on healthy diet principles Incentives for healthy food production Tax on unhealthy foods Subsidies for healthy food	-	Facilitate access of healthy food to markets and institutions Raise awareness and promote healthy food to food operators and consumers Strengthen local production	Cooperation with food operators to produce food with pro-health values	Support culture and consumption of wholesome food
4	**Food safety system establishment - GAP, GMP, GHP- HACCP**	Establishments of FS system: Monitoring Control Training Research	-	Encourage and educate food operators for HACCP implementation Monitor and assess risk Inspectoral Control System	-	Increase application of HACCP systems among food operators

Agricultural measures to address food security in vulnerable population

Strategies to support agricultural productivity in ensuring food security for vulnerable groups - children, elderly, poor and disabled were found in 4 policies. The Bulgarian, Georgian and Moldavian policy measures mainly propose the development of mechanisms to support agricultural production in rural settings and facilitated access (e.g. through subsidies) of healthy foods (e.g. fruits and vegetables or fish) to vulnerable groups. Beside this, there are programs such as free milk and fruits for school children in Bulgaria and healthy foods in schools in Georgia or self-supply anti-crisis programs in Slovenia.

Measure to address problems of micronutrient deficiencies
The Bulgarian and Moldavian policies propose a fortification of staple foods with iron, folic acid and iodine. Slovenia encourages production of food for those with special nutritional needs, while Georgia asks for agricultural response to micronutrient deficiencies. None of the countries considers methods for bio-fortification as possible solution to address micronutrient deficiencies widely. The issue of fortification of staples in many of CEE countries is at the control of the state and is not regulated by the law i.e. law only regulates salt iodisation. The domestic food industry is commonly believed to lack technological and other knowledge and incentives to embark into these processes.

Promotion of food with health- benefit -production, processing, trade and distribution
Strategies to promote the production of healthy foods, facilitated access to the markets and education of producers and consumers were found in 4 policies. They are comprised of (i) Proposed activities to rise and approve awareness on healthy food consumption (in Bulgaria, Slovenia and Moldova), (ii) Giving incentives for healthy food production such as: subsidies for production (Bulgaria), facilitation of access to the markets, strengthening of local production (Slovenia), cooperation with food operators for development of pro-health food products (Poland).
There are few successful examples from other CEE countries (i.e. Poland, Czech Republic and Romania) which set certain taxes on fat (butter and snacks) and sugar (sweets, ice creams, confectionary) that had a huge impact on reduction of consumption of these commodities. Fruit and vegetables prices reduction at retailers has shown to be effective pricing strategy, as well (EHN, 2011).
However, agricultural policies in these countries have been tailored to produce cheap energy food supply with low nutritional profiles – low priced animal products with undesired fats and highly refined cereals, which were to satisfy basic needs of public and keep social ease. However, these policies have dire health consequences, reflected in rampant overweight, obesity and non-communicable diet-related diseases (WHO, 1998).

Food safety system establishment
The Bulgarian and Slovenian policies propose an integrative approach to establish a food safety monitoring system. This entails education and training on implementation of HACCP principles, assessment of risks, recommendations, monitoring, research (Bulgaria) and enforcing of inspectoral control system (Slovenia). Moldova however, still encourages food producers to implement HACCP systems in their processes. This situation clearly shows significant difference in development of food safety system establishment between those countries in EU and those countries which are still striving toward EU. Since implementation of food safety system entails financial, organizational and other resources such as well trained staff, it presents significant investing challenge for a food operator and a state and the whole process is slow. It is also important to mention that, while Western Balkan countries are building EU compatible regulatory systems, former soviet countries - Commonwealth of Independent States, use principles of Russian GOST “Gosudarstvennyy standart” system, which is now administered by the Euro-Asian Council for Standardization, Metrology and Certification, a standards organization chartered by the Commonwealth of Independent States which are not compliant to the WTO standards and are not recognized by most of the world’s trading countries (Mizik, 2010).

Other strategies
There are some other aspects projected in a few policies. For example, the policy of Georgia emphasizes the importance of development of monitoring systems for food availability and food consumption. Capacity Development Network for CEE countries recognizes the need for these systems and it can be expected that it will come up with their own databases and information systems which will help them to develop future policies (Pavlovic *et al.*, 2009).
The Slovenian policy stimulates development of local sustainable supply systems and economies and rural development and points out importance of consumer needs’

satisfaction. It is the only policy that recognizes the importance of preservation of local sustainable supply systems. While other countries have even more natural resources to do so, their traditional diets are vanishing and local commodities are being replaced with intensively imported foods e.g. tropical fruits in continental countries or intensively raised high-cropping easy-storing varieties of fruits and vegetables with lower content of vitamins or breeding of farm poultry with higher percent of fat and farm fish with lesser percent of poly-unsaturated fatty acids (EHN, 2011).

CONCLUSION

Strategies aimed at the agricultural sector for better diets in CEE countries' nutritional policies all together imply mechanisms such as production promoting mechanisms, nutrition education on different levels, market accessibility and regulation of food safety systems. The low number of available policies is an important indicator of the state at which countries of the region are in term of their capacity and resources to formulate and then take over proposed actions. Specific settings of the countries and historical background and development have to be taken into consideration. Nevertheless, there are prerequisite measures that are of crucial importance for further development – raising awareness of the important link between agriculture and nutrition among professionals from various fields-agriculture, nutrition, food technology, politics, economics, market, civil society etc. and bringing them together around building of capacity to tailor appropriate policy which will steer agricultural activities towards meeting nutritional needs for all.

ACKNOWLEDGEMENTS

This work was completed in collaboration with Department for Food safety and Food quality, Faculty of Bioscience Engineering, Gent University, as a part of low and middle income countries nutrition policy review. It was supported by Centre for Research Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade.

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MICROBIOLOGICAL VERIFICATION OF SANITATION PROCEDURES IN MEAT ESTABLISHMENTS

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ABSTRACT: The purpose of maintenance of hygiene is removing of all hazards that could result in food contamination; thus, its performance should be monitored and controlled. Direct methods of sampling of working areas, like swab method, are often used for detecting microorganisms presented on the working areas in meat industry facilities. The aim of this research was to evaluate the efficiency of the applied sanitation procedures in meat establishments. In this paper, microbiological safety of equipment, tools and working areas after disinfection during the certain steps of the technological procedure of meat processing and production was investigated. Swabs were taken from the same slaughterhouses during monthly routine controls from 2009 until 2011. Microbiological analysis were performed and following microorganisms were tested: *Salmonella* spp., *Escherichia coli*, coagulase-positive *Staphylococcus*, sulphidoreducing *Clostridium*, *Enterobacteriaceae,* as well as the total number of bacteria. From the total of 100 swabs taken from the working areas, tools and equipment in each year of research, 83% and 97% of the results in 2009 and 2011, respectively, were graded as acceptable. Comparing the period from year 2009 until 2011, the reduction trend of the number of unacceptable samples based on microbiological criteria is evident. This type of trend confirms that application of good manufacturing and hygienic practice is necessary to maintain high standard of hygienic and safe products.
Key words: *microbiological safety, hygiene of manufacturing plant, meat industry*

INTRODUCTION

Impurities, food residues and other waste materials attract pests and represent potential source of microbiological and physical contamination. Regular and thorough cleaning is necessary to remove impurities and maintain the facilities and equipment in a clean condition. Verification of the effectiveness of cleaning, washing and disinfection procedures as an addition and support to the process hygiene criteria in food production is achieved by analyzing the samples from the surfaces on certain microorganisms. While defining the areas to be sampled, the area sampling frequency, as well as eligibility criteria and possible corrective measures, the subject must take into account all available information about the potential dangers that can occur in the stages which are under its control.

In food industry, including production facilities for meat processing, there is a high risk of microbial contamination. In order to prevent the spread of contamination and risk to the human health, it is necessary to comply with good manufacturing practice (GMP) and good hygiene practice (GHP) and implement permanent control of production hygiene by taking all measures that help reduce risk, and economic damage in the meat industry (EU Concil Directive on the hygiene of foodstuffs, 1993).

In terms of microbiological safety, during meat production and processing, of particular significance are the following phases: bleeding, skinning/scalding, evisceration, cooling, deboning and grinding (Plavšić et al., 2007). Lack of skilled labor may contribute to the emergence and spread of contamination, leading to unsafe product which may represents a risk to the human health. Careless performance of bleeding and skinning can lead to meat surface contamination by impurities, therefore with the microorganisms (Bem and Adamič, 1991). Microorganisms like *Salmonella* species, *Escherichia coli*, *Proteus* species,

Clostridium species and others are regular inhabitants of animal intestines. However, their presence on other areas like meat surface is regarded as microbial contamination and possible cause of alimentary toxicoinfections and diseases. *Salmonellae* spp. is often pathogenic for humans and cause intestinal disease salmonellosis. *Clostridium* species are toxigenic and sporogenic microorganisms and may lead to serious toxicoinfections. *Escherichia coli* is indicator of fecal contamination, produce enterotoxins and cause enteritis (Plavšić et al., 2010; Plavšić et al., 2009; Plavšić et al., 2007). This indicates that in the stages of slaughter and carcass dressing, microbial cross-contamination may occur, either directly or via equipment, especially when the principles of GMP/GHP and HACCP are not followed (Malakaukas et al., 2006). Therefore, it is important that the principles of hygiene are met at every stage of the technological process of meat production.
New EU legislations concerning food safety regulate each level of food chain and describe the specific rules for food product of animal origin (Taylor and Holah, 2000). In order to assess the risk and take appropriate measures, control of hygiene facilities like equipment and tools, must be accompanied with the microbiological checking of hygiene maintenance by taking swabs (Barlow et al., 2002). With the implementation of HACCP principles, evaluation and control of hazards significant for food safety is performed. According to EC Regulation No 2073/2005, and with the implementation of HACCP programs, microbiological testing of hygienic status of carcasses, working surfaces, tools, supplies, equipment and machinery are provided (Commission regulation EC, 2073/2005).
The aim of this research was evaluation of the efficiency of the applied hygienic procedures on microbiological safety of equipment, tools and working areas after disinfection during the certain steps of the technological procedure of meat processing during the period of 2009 until 2011.

MATERIALS AND METHODS

The research included two facilities for meat production and processing during years 2009, 2010 and 2011. Five swabs were taken in each facility on a monthly basis during ten months. Surfaces from which swabs were taken were a table for cutting, knife, meat chopper, meat grinder and containers for meat disposal. Swabs were taken after sanitation procedure was performed. For taking the swabs, sterile set swabs with the test tube with 10 ml of sterile saline peptonic solution with neutralizer and metal templates (dimension 10x10cm) were used. Swabs were taken according to SRPS ISO 18593 (2008). Microbiological analysis of swab samples included following microorganisms:

- *Salmonella* spp
- *Escherichia coli*
- Coagulase-positive *Staphylococcus*
- Sulphitoreducing *Clostridium*
- *Enterobacteriaceae*
- Total number of microorganisms

Determination of these microorganisms was conducted using ISO standards (SRPS EN ISO 6579,2008; SRPS ISO 16649-2,2008; SRPS EN ISO 6888-1,2003; ISO 15213,2003; SRPS ISO 21528-2,2009; SRPS EN ISO 4833,2008)

RESULTS AND DISCUSSION

To check the hygiene of the facilities for production and sale of food, samples are taken from surfaces that are washed and disinfected. Since it is not defined in Regulation, the method, sampling frequency, the selection of microorganisms, and acceptance criteria should be outlined in the plan of self-control for food business operator. The guide for application of microbiological criteria for foods, chapter V, presents the microbiological criteria for objects,

surfaces and hands coming into contact with food, as a recommendation for food business operators (www.mpt.gov.rs).
In most cases, surface hygiene assessment is based on the number of microorganisms per cm^2 surface. In some cases, the specific bacteria such as *Escherichia coli* (an indicator of fecal contamination), *Staphylococcus aureus* (commonly found on the hands), *Salmonella* spp, *Enterobacteriaceae* are determined. Recommendations of national legislations are that if the contact surfaces have total number of microorganisms of 0-10 cfu/cm^2 and number of *Enterobacteriaceae* of 0-1 cfu/cm^2 they are evaluated as acceptable. Surfaces with total number of microorganisms and number of *Enterobacteriaceae* above the mentioned values are evaluated as unacceptable. Table 1 shows the results of swabs analyses during three year period (2009-2011).

Table 1. The percentage of acceptable and unacceptable results of analyzed swabs according to recommended hygiene criteria

Year	Total number of swabs	Acceptable	Unacceptable
	n	%	%
2009	100	83	17
2010	100	91	9
2011	100	97	3

Reasons for microbiological unacceptability of some swab samples were increased total number of microorganisms, increased number of *Enterobacteriaceae* and presence of *Escherichia coli* (table 2). During year 2009, 17% of swab samples were unacceptable due to increased total number of microorganisms, from which 14% of the samples had increased number of *Enterobacteriaceae,* and 11% of samples had *Escherichia coli* isolated. Detection of pathogenic microorganisms in the next two years tended to decrease. During year 2010 *Escherichia coli* was isolated from 7% of the tested samples, while 9% of total tested swab samples were unacceptable due to increased number of *Enterobacteriaceae* and total number of microorganisms. In year 2011 3% of the total tested swab samples were unacceptable due to the increased number of *Enterobacteriaceae* and total number of microorganisms. *Salmonella* spp, coagulase-positive *Staphylococcus* and sulphitoreducing *Clostridium* were not isolated in any of examined sample.
Total number of microorganisms in unacceptable samples was in the range of 60 to $2.1x10^3$ cfu/cm^2. Total number of *Enterobacteriaceae* in unacceptable samples was in the range of 20-190 cfu/cm^2. Number of *Escherichia coli* in unacceptable samples was in the interval from 35 to 100 cfu/cm^2. Since the swabs were taken from the clean surfaces after disinfection, findings and the presence of isolated microorganisms suggest that performed sanitary procedures were not efficient enough to remove microbial contamination.

Table 2. Results of analyzed unacceptable swabs (presence of microorganisms in % of samples)

Microorganisms	Year		
	2009[a]	2010[a]	2011[a]
Salmonella spp / cm^2	ND*	ND	ND
Coagulase-positive Staphylococcus / cm^2	ND	ND	ND
Sulphitoreducing *Clostridium* / cm^2	ND	ND	ND
Escherichia coli / cm^2	11%	7%	ND
Enterobacteriaceae / cm^2	14%	9%	3%
Total number of microorgansms /cm^2	17%	9%	3%

Legend: ND* - not detected
[a]The total number of examined swabs was 100 per year

Escherichia coli, as well as other *Enterobacteriaceae* are indicators of fecal contamination and suggest on insufficient hygienic performance of slaughter and carcass dressing operation. Stages of production such are dehiding and evisceration, if performed unprofessionally, may lead to contamination of meat, and also equipment and tools that are

in contact with meat, with microbial indicators of fecal contamination. During further stages of deboning and grinding, present microorganisms may multiply if these operations are performed unhygienically and without the maintenance of the cold chain, leading to further cross-contamination of the meat.
Introduction of HACCP principles, hazard analysis in each phase of production process, determination of the critical control points and implementation of control measures for management of the risks in meat production, are ultimately preconditions to safe meat production and hygienic and safe final product.

CONCLUSION

Reduction of the number of unacceptable swab samples of working surfaces in examined meat establishments indicates improvement of hygienic measures during the production process. Based on the fact that a large number of pathogenic microorganisms in the meat chain originate from the primary production or are introduced during meat processing, absolute imperative for improvement the safety of the meat and meat products is that meat establishments must comply with process hygiene criteria and good manufacturing and hygiene practice.

ACKNOWLEDGEMENT

These results are part of project TR-31055 supported by the Ministry of Education and Science of the Republic of Serbia.

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SUSTAINABLE DEVELOPMENT OF THE RIVER SAVA ECO-SYSTEM FROM THE ASPECT OF CONTAMINATION BY POLYCHLORINATED BIPHENYLS (PSBs)

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ABSTRACT: Environment protection, rational utilization of natural resources, reduction of pollution and use of natural resources, in a way to preserve and make them available for future generations, are some of many goals of sustainable development concept. The burden imposed on river ecosystems by polychlorinated biphenyls (PCBs) has great impact on sustainable development of our rivers. Properties of PCBs (resistance to photolytic, biological and chemical degradation) provide their broad distribution within the aquatic eco-system. Exposition to PCBs can cause harmful effects on aquatic biodiversity and, consequently, on animal and human health.
Objective of our study was to determine the level of contamination of fish by PCBs as bio-indicators of the contamination of the aquatic eco-system of river Sava.
In the period September-December 2011, 75 samples of river fish were collected (carp, crucian carp, pike, pike perch, bream and sturgeon) in the river Sava, upstream and downstream from Belgrade. Polychlorinated biphenyls were determined by GC/ECD on Thermo TG5MS capillary column (30m x 0.25mm i.d. x 0.25µm film thickness). Method detection limit is 0,001 mg/kg.
In the investigated fish samples the quantities of PCB residues ranged from 0,002–0,246 mg/kg (mean value 0,023 mg/kg). Compared to values obtained for 75 sea fish samples (hake, mackerel, sprat, sea bass, sea bream, etc), studied in 2011, where quantities of PCB residues ranged from 0,001-0,104 mg/kg (mean value 0,006 mg/kg), quantities of PCB determined in fresh water fish were four times higher, compared to sea fish.
The obtained results indicate that, the eco-system of river Sava, on the territory of the city of Belgrade, is compromised, and consequently it is necessary to implement measures for environment protection. These measures include strict prohibition of disposal of PCB into the river, collection and safe disposal of utilized oils (machine oil, transformer oil and oil used in the electronic industry, etc.), according to the National Strategy of Sustainable Development of Republic of Serbia.
Key words: *sustainable development, polychlorinated biphenyls (PCBs), fish, river Sava*

INTRODUCTION

The concept of sustainable development has become one of the most important concepts of our time. As multi-dimensional phenomenon, sustainable development has goal to integrate economic, ecological, social and institutional subsystems into a whole, taking care of their mutual influence. Environment protection, rational utilization of natural resources, reduction of pollution and use of natural resources, in a way to preserve and make them available for future generations, are some of many goals of sustainable development concept (Castro, 2004; Sarcevic et al., 2009). As it was said in the National Strategy of the Sustainable Development of the Republic of Serbia, every generation have same chance for using resources and healthy environment (Službeni glasnik RS, br. 55/05, 71/05 –ispravka 101/07). Realization keys for sustainable development concept are recongnized in prediction and prevention of risks in all areas of activity (Baras and Matekalo-Sverak, 2003; Sarcevic et al., 2011). Indicators of sustainable development include parameters used to monitor causes and consequences of human action of biosphere in order to find answers, i.e. establish actual activites which shall prevent the adverse impact (Baras et al. 2007; Baras et al 2004).
The burden imposed on river ecosystems by polychlorinated biphenyls (PCBs) has great impact on sustainable development of our rivers. Properties of PCBs (resistance to photolytic,

biological and chemical degradation) provide their broad distribution within the aquatic eco-system. Exposition to PCBs can cause harmful effects on aquatic biodiversity and, consequently, on animal and human health. Main concern is address to persistence and bioaccumulation through the food chain and their toxic effects (Janković et al, 2009). The main human intake of PCBs is by food consumption and particularly through consumption of fish. Fish consumption contributes up to 50% of intake of PCBs. Accumulation of PCBs in fish depends on their concentration in water, than on life stage, species and fat content of the fish (Bayarri et. al. 2001; Bordajandi et. al. 2001).From that point of view aquatic fauna might be used as indicator organisms for the evaluation of pollution of the aquatic environment with PCBs.

Mixtures of non-dioxin-like PCBs are assessed on the basis of analysis of the six so-called "indicator PCBs" - 28, 52, 101, 138, 153 and 180. These congeners are selected as suitable representatives for all PCBs because they are dominantly present in biotic and abiotic matrices.

Objective of our study was to determine the level of contamination of fish by PCBs as bio-indicators of the contamination of the aquatic eco-system of river Sava.

MATERIAL AND METHODS

In a year 2011, six different fish species have been collected from the Sava River: pike (*Esox lucius*), bream (*Abramis brama*), crucian carp (*Carassius carassius)*, pike pearch (*Stizostedion lucioperca*), sturgeon (*Acipenser sturio*) and carp (*Cyprinus carpio.*). Sampling points were separated in two locations: upstream and downstream from Belgrade.

Fish specimens were kept frozen at -20 °C before analysis. Samples were chopped into 2-3 cm thick portions and homogenized. In the fat extracted from sample with petrol-ether were determinated non–like dioxin PCB by gas chromatography.

Gas chromatograph GC Varian Model 3800 equipped with a ^{63}Ni electron capture detector (ECD) and Thermo TG5MS column (30m x 0,25mm i.d. and 0,25μm film thickness) were used for analysis of PCBs. Operating conditions were as follows: injector 250°C; detector 300°C; column oven program: initial 50°C raised to 200°C at 50°C/min, hold 2 min then raised 215°C at 2.5°C/min, hold 5 min and finally raised to 230°C at 2°C/min, hold 9,5 min. The highly purified nitrogen carrier gas flow was 1 ml/min. Data acquisition was performed by Varian Star software.

Analytical quality control was achieved by using certified reference material ERM-BB446 (IRMM, Belgium). Trueness and intermediate precision were fulfilled with specific requirements for determination of non dioxin-like PCBs (Specific requirements for determination of non dioxin-like PCBs, 2008).

The concentrations of target individual congeners (IUPAC numbers 28, 52, 101, 138, 153 and 180) were expressed in nanogram per gram on a fresh weight. PCBs congener IUPAC number 116 was used as an internal standard. The results are expressed as mean value ± Sd.

RESULTS AND DISCUSSION

The content of non dioxin-like PCBs in fish samples from Sava River is shown in table 1. PCB concentrations in fish from Sava River were in the range of 2 - 246 ng/g (mean 23 ng/g). Content of PCB in all samples were below the maximum residue limits (MRL) established by Serbian legislation - 3000 ng/g fresh weight (Pravilnik o količinama pesticida, metala i metaloida i drugih otrovnih supstancija, hemioterapeutika, anabolika i drugih supstancija koje se mogu nalaziti u namirnicama, 1992) as well as USA MRL – 2000 ng/g (Rockville, 2001). No maximum levels for non dioxin-like PCB in feed and food have been set in European Union. Several Member States have national provisions in place as regards to maximum levels for non dioxin-like PCB in foodstuffs. For

foodstuffs Belgium and France established maximum levels for the sum of seven indicator PCBs of 100 or 200 µg/kg fat, depending on the type of product. In the Czech Republic, maximum levels for the sum of seven indicator PCBs for various foodstuffs have been set in the range of 50-5000 µg/kg fat (AOAC, 1990). Content of non dioxin-like PCBs in our study, taking into consideration average fat content of examined fish species, is lower than levels mentioned above. Maximum levels for total PCBs established in Community legislative from 2006. were based on toxic equivalency factors (TEFs) and MRLs is expressed as sum of dioxins and dioxin-like PCBs and don't include non-dioxin-like PCBs (EC2006, 2006).

Table 1. Content of non dioxin-like PCBs in Sava fish (ng/g fresh weight)

Species	No	Σ PCBs ngg^{-1}		
		range	mean	**Sd**
crucian carp	12	5 - 31	17.6	7.3
pike pearch	12	2 - 21	12.5	4.8
carp	12	4 - 246	64.1	59.1
bream	15	6 - 40	18.6	9.7
sturgeon	12	5 – 28	17.9	6.6
pike	12	5 - 11	9.1	1.6
Σ	75	2 - 246	23.3	16.8

PCB concentrations in fish from Sava River were in the range of 2 - 246 ng/g (mean 23 ng/g). Content of PCB in all samples were below the maximum residue limits (MRL) established by Serbian legislation - 3000 ng/g fresh weight (Pravilnik o količinama pesticida, metala i metaloida i drugih otrovnih supstancija, hemioterapeutika, anabolika i drugih supstancija koje se mogu nalaziti u namirnicama, 1992) as well as USA MRL – 2000 ng/g (Rockville, 2001). No maximum levels for non dioxin-like PCB in feed and food have been set in European Union. Several Member States have national provisions in place as regards to maximum levels for non dioxin-like PCB in foodstuffs. For foodstuffs Belgium and France established maximum levels for the sum of seven indicator PCBs of 100 or 200 µg/kg fat, depending on the type of product. In the Czech Republic, maximum levels for the sum of seven indicator PCBs for various foodstuffs have been set in the range of 50-5000 µg/kg fat (AOAC, 1990). Content of non dioxin-like PCBs in our study, taking into consideration average fat content of examined fish species, is lower than levels mentioned above. Maximum levels for total PCBs established in Community legislative from 2006. were based on toxic equivalency factors (TEFs) and MRLs is expressed as sum of dioxins and dioxin-like PCBs and don't include non-dioxin-like PCBs (EC2006, 2006).
Contents of non dioxin-like PCBs in freshwater fish presented in the recent studies published in literature differ significantly between themselves.
Similar results to ours were published by Vojinovic-Miloradov et al. 2002 in regard to Danube fish perch, carp and pike – 6-22.5 ng/g (Vojinovic-Miloradov et al. 2002), Bosnir et al. 2005 in different fish species from Sava river - 8-177 ng/g (Bošnir et. al 2005), Mazet et al. 2005 in fish from Drome river (France) - 7,8-56,9 ng/g (Mazet et al. 2005) and van Leeuwen et al. 2007 in pike perch from Netherlands river - 37-87 ng/g (van Leeuwen et al. 2007).
Higher content than in our study were presented in Bazzani et al. 1997 in roach fish from river Arrone (Italy) - 1075,6 ng/g (Bazzanti et al. 1997) and Zhao et al. 2007 in crucian carp from China, up to 1451 ng/g (Zhao et al. 2007).
Vives et al. 2005 in brown trout from Redo lake in Spain founded 8.22 ng/g (Vives, et al. 2005) and Falandysz et al. 2004 - 3.8 ng/g non dioxin-like PCBs in perch from Odra river (Falandysz et al. 2004), which are lower content of PCBs than levels presented in our study.
In order to assess the level of contamination of the River Sava's eco system through fish, we examined 75 samples of six marine fish, most important in the diet of Serbian population: hake, mackerel, sprat, sea bass, gilthead and scorpanea. PCB concentrations in marine fish were in the range of 1- 104 ng/g (mean 6 ng/g).

The fact that average PCB value in fresh water fish is 4 times higher compared to sea fish indicates the problem of contamination of Sava's eco system as well as that the ecosystem of the Sava River in Belgrade area is at risk and the necessary measures for environmental protection should be implemented.

CONCLUSIONS

The obtained results indicate that the eco-system of river Sava, on the territory of the city of Belgrade, is compromised, and consequently it is necessary to implement measures for environment protection. These measures include strict prohibition of disposal of PCB into the river, collection and safe disposal of utilized oils (machine oil, transformer oil and oil used in the electronic industry, etc.), according to the National Strategy of Sustainable Development of Republic of Serbia.

ACKNOWLEDGEMENTS

This work was supported by the project III 46009, financed by the Ministry of Education and Science of Republic of Serbia.

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ANTIBIOTIC RESISTANCE IN THE BREEDING OF WARM WATER FISH

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ABSTRACT: In Serbia there are about 17 ha for growing trout pond and 8,940 ha for the cultivation of carp and other warm-water fish. According to the statistical data, on these production areas the production in 2010 amounted to about 7,000 tons of carp fish and 1,200 tons of fish species. The main obstacle in the chain of intensive fish production rests on the bacterial disease, somewhat less parasitic and virology etiology kind. Reproduction and growth of bacteria in adverse growing conditions (insufficient water changes, presence of toxic gases, overcrowding of fish per unit area, low hydro reclamation and zoo hygienic conditions in the preparation of facilities for hatching), causes the disease. Conditional pathogenic bacteria from *Aeromonas* are common causes of disease. Due to a lack of monitoring and laboratory examination, the antibiotics used to combat bacterial diseases are used without an established antibiograms and often inadequate doses. All of these factors are detrimental to the development of resistance of the applicable antibiotics. Sensitivity analysis of isolates to antimicrobial drugs showed the presence of significant resistance to Flumequin and Oxitetraciklyne over 35% to 40%, while resistance to Oxitetraciklyne is close to 20% of examined cases. The resistance to Flumequin is still very low and resulted in only 2 %.These findings show that the antimicrobial drugs are used in inadequate manner and in the future it can be expected to further increase resistance and reduce the number of effective drugs. Considering that microorganisms are in question *(Aeromonas hydrophila)* which can endanger public health, the contagion especially in the case of reduced sensitivity to antibiotics can result in the danger to people.
Key words: *fish, antibiotics, resistance, environment*

INTRODUCTION

The widespread use of antimicrobial agents and the ability of microorganisms to adapt to unfavorable environmental conditions have led to the emergence and spread of resistance of pathogenic bacteria in animal production worldwide (Levy, 1998; Witte, 1997). Some bacteria have become resistant to most or even all of the antibiotics and chemotherapy, which justifies the fear of the return of mankind in the age before antibiotics and the possible development of fatal infectious diseases (Asanin et al., 2001). The use of same or similar chemical and antimicrobial means for the people, animals, and plants contributed to the appearance and the expansion of bacterial resistance. The appearance of bacterial resistance which endangers the production of fishing is confirmed in the studies (Plavsa et al., 2001; Plavsa et al., 2007; Stojanov et al., 2010). Bacterial illnesses of the fish are very important in the overall breeding phase; in addition, they are a main problem for a successful fishing business. Moreover, bacterial illnesses are one of the limiting factors in the overall development in the fishing industry and they secure very quality and demanding fish meat. In the Republic of Serbia, 17 ha of water surface are designated for the breeding of trout while the surface for breeding warm water fish is 8,900 ha. According to the statistical data, the production on this water surface in 2010 was 7,000 tons of carp and 1,200 tons of other kind. Consumption of fish meat in Serbia is around 32 million kg on annual basis. Only one third of that is produced domestically but the rest is imported. The geographical position of Serbia is ideal for breeding freshwater fish; only China and Israel share the same position. Serbia is located at the latitude 40.0 and it has identical climate as Peking, a location that produces the most fish in the whole world. Considering the given facts and the advantages of breeding and producing fish meat, and that the Republic of Serbia imports 25 million dollars of fish

annually, it is reasonable that fishing experts and the state organize and intensify the production of fish meat. Along side the pathogenic causes of emerging fish illnesses; changing environment has a significant effect. The water in which fish lives is rich in organic material of different origin because of increasing number of fish per unit area and more nutrition. This kind of environment is susceptible for the development of pathogenic and conditionally pathogenic bacteria. Being that most fisheries are poorly maintained in the sense of implementation of mandatory preventive veterinary-sanitary measures, limited water supply for fisheries, optimal plantation density and the quality of planting materials, bacterial diseases can not be solved only by antibiotics. Treatment of exhausted and parasites loaded fish in the late stage of disease often results in poor outcome and large economic losses. The causes of bacterial diseases for fish are usually from Aeromonas group *(A. Hydrophila, A. Salmonicida, A. Punctata, A. Sobria*) and *Pseudomonas* (*P. Fluorescens*). Although these bacteria are known as pathogenic some of them may be normal inhabitants of the red flora in healthy fish. It is known that due to stressful situations such as high water temperature, low oxygen level, accumulation of waste products, especially ammonia, high-density plantations and others, these bacteria can modify virulence and contribute to high mortality rate. Negative relationship between these factors results in stressed organism of the fish; and the presence of pathogenic and conditionally pathogenic agents exert their effect when they are in greater concentration in a weakened organism. For these reasons, epizootic (eritrodermatitis, carp pseudomonosa, silver carp skin disease, columnaris disease) caused by bacteria erupts in fisheries. The key to successful treatment of bacterial infections in the early detection of the disease is accurate diagnosis and implementation of appropriate therapy. The most effective therapeutic means used via pellet to treat bacterial diseases of fish is antibiotics (Jovanovic et al., 2000, Plavsa et al., 2001). Necessary efficacy of administering oral medicine is achieved by the timely application of bacteriological diagnosis and effective antibiotics based on antibiogram results. If not treated in time, the disease spreads and the appetite of the fish decreases, all of which reduces the chance of successful treatment. Remedy must be accurately metered and the length of treatment depends on the intensity of disease and water temperature. The most commonly used antibiotics to treat bacterial diseases of fish are Oxitetraciklyne, Flumequine, Fluorphenicol, Olaqvindox. The most serious problem in the treatment of bacterial diseases is the emergence of resistance to the medicine. The resistance of microorganism occurs most commonly if the chosen antibiotic is given in smaller doses than prescribed (often very difficult because of the assessment of fish in large space) and a shortened period of time.
Our research is aimed at determining the extent and the presence of bacterial flora in carp ponds in Vojvodina, the effect of antibiotics on the isolated bacteria, and incidence of resistance to conventional antibiotics.

MATERIAL AND METHODS

The study material was represented by samples of carp from the pond where they were diagnosed with skin lesions and gills. Isolation of Aeromonas was carried out by direct plating of samples onto the nutrient medium (Trypticase soy agar with 5 % defibrinated sheep blood) and selective base McKonkey agar (Quin, 2002). Bases are incubated for 24 hours at 37 degrees Celsius. Identification of Gram-negative bacteria, which created a clear or slightly less hemolysis, was done by determining the physiological characteristics of isolates (API strip). Susceptibility testing of Aeromonas to antimicrobial drugs has been conducted with disk diffusion method on Mueller-Hinton plate. (Quin, 2002, NCCLS). Zone of inhibition appeared around each disc were measured and the sensitivity is determined in relation to the diameter of which appeared around the discs of antimicrobial drugs. The study was conducted on the basis of instructions (NCCLS), so that the zone diameter of less than 13 mm interpreted as a resistance (R), zone of 15-16 mm as intermediate (I), and zone of 17 mm or more as sensitive (S). The sensitivity of the isolates was tested against the following antimicrobials: Fuorphenicol, Flumeqvin, Olaqvindox and Oxitetraciklyne.

RESULTS AND DISCUSSION

The study was conducted in the 2010-2011 period. During this period a total of 175 clinical specimens were examined, of which 78 samples had changes in the skin and gills and all were isolated from Aeromonas hydrophila. Changing parts of the skin, necrotic and hyperemic edges in the middle, were the size of a few mm to over 20 mm (Table 1). During the susceptibility testing of isolates on antimicrobial drugs, with disk diffusion method, drugs commercially available in the market (Fluorphenicol, Flumequine, Olaquindox and Oxitetraciklyne) were used.

Table 1. Antimicrobial susceptibility testing of Aeromonas hydrophila isolated from skin changes of carp

	Antimicrobial drugs		Carp with skin changes	
		O (%)	I (%)	R (%)
1.	Flumequine	48,99	14,31	36,70
2.	Olaquindox	50,12	30,87	19,01
3.	Oxitetraciklyne	13,70	42,60	43,70
4.	Fluorphenicol	65,34	31,96	2,17

In our studies we found that the resistance to Flumeqvin Oxitetraciklyne was 36% and 43% respectively (for clinical specimens of diseased fish) and Olaqvindox as much as 19%, indicating multiresistantance to most antibiotics. Castro -Escarpulli et al., (2003) found that the best antimicrobial effect is achieved by applying the first-generation quinolone and the second and third generation cephalosporin and our studied showed high resistance to flumequin percentage (36%) and Olaqvindox (19%) as representatives of the quinolone. The least resistance is expressed in fluorphenicol with 2.17% percentage. In earlier studies (Plavsa et al., 2001, 2007) it was found that for 4 years the number of resistant type of *Aeromonas hydrophile* on Oxitetraciklyne increased from 21.7% to 77.2%, while that change for Flumeqin ranged from 12.7% of resistant type to 53.1% after 4 years. This increase of resistant types on mentioned antibiotics is a consequence of several important factors. These were the relatively low specific doses or doses that are translated by the quantity of food. It is known that sick fish has drastically decreased appetite, so that the amount of regular food intake and food with built in drug are reduced. On the other hand, in fisheries, unskilled professionals are often involved in the treatments causing multi-link and long-term damages. In last few years the problem of resistance has been solved by increasing the drug dose however that practice has no medical or economical perspective. The tendance of beta hemolozin in strains *A. Hydrophila* is not just in the relation with the multiresistance but also with virulence strains of this bacterium as indicated by the results (Daskalov, 2006). Interconnection between these characteristics is not only a medical problem for fishery but also for human population.

CONCLUSIONS

Fish diseases have always represented a significant problem of fisheries production, but in recent years by intensifying production, fish diseases especially bacterial etiology, have become a far more significant problem. These diseases are often present during breeding in both one-and-two year carp fish. The choice of antibiotics is smaller but the resistance is becoming a growing problem. All of this points to the need for more rigorous implementation of preventative and zoo hygienic measures. The study shows *Aeromonas Hydrophila* present in all processed carp sample signified with clinical changes on the skin and gills. Sensitivity analysis of isolates to antimicrobial drugs showed the presence of significant resistance to Oxitetacyclin, over 43% and Flumequine over 36%. Without improving the veterinary-sanitary conditions, regular examinations and early diagnosis, this disease along with other fish

diseases, represent a growing problem in the near future and will be a limiting factor in the development of fisheries.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project “31011” financed by the Ministry of Science and Technological Development, Republic of Serbia.

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INGREDIENT PROFILE AND NUTRITIVE VALUE: CORN DRIED DISTILLERS' GRAINS (DDGc) VS. CORN DRIED DISTILLERS' GRAINS WITH SOLUBLES (DDGSc)

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ABSTRACT: The aim of this study was to characterize and conduct comparative analysis of corn dried distillers' grains with / without solubles (DDGc and DDGSc). These two animal feedstuffs are the main by-products of bioethanol industry. Representative samples of these subproducts were taken from the largest Bulgarian distilleries and analysed for DM, CP, EE, CF, NFE, ash, nutritive value (energy and protein nutritive value), aminoacid profile (relative and absolute value) and mineral content (macro- and microelements). The chemical analyses were made following AOAC, 2002. The nutritional assessment of both feeds included calculation of energetic and protein values by total or particular chemical composition. Based on the results of the conducted analyses it was concluded that corn dried distillers' grains with solubles (DDGSc) is superior to corn dried distillers' grains (DDGc). Potential correlations should be searched between protein (CP, PDI and BPT) and energy (FUG and FUM) nutritional value of both feeds and the expected animal performance.
Key words: *DDGc / DDGSc, nutritive value, amino acids, energy, protein*

INTRODUCTION

In recent years, following EU and global trends, in Bulgaria were put into operation some large bioethanol distilleries using cereals as raw material.
A revival of this branch is favored by accession of the country to the EU, subsidy programs, so-called "Green Thinking" boom and the production of biofuels from renewable materials. The main product in bioethanol industry is bioethanol and subproduct – distillers' grains (SC). Corn distillers' grains (DGc), in all its forms, represent a main by-product of ethanol production from corn grains. This byproduct obtained in plants producing bioethanol for biofuels as well as in the process of industrial production of ethanol and alcoholic beverages. Currently in this sector are accepted and standardized two basic manufacture methods - dry and wet distillation. The main manufacture method in Bulgaria is dry grinding of grains.
Based on the proportions of our country, current situation, an ad hoc policy, the fragmentation of our husbandry and smallcapacity farms (in size and number of animals). So it is established that dried version of the DCc (DDGc and DDGSc) is more applicable.
These products don't have discermable features and the difference between them is somewhat fuzzy and unclear in spide of the existing definitions for each particular by-product. In addition the amount of distillers' soluble inputs in the production of DDGSc varies within a wide range. Many scientific reports failed to present data about the nature of the feeds – wether they were with / without solubles. So actually DDGc often is identified with DDGSc [Schingoethe, 2006].
However, a review of available literature data about chemical composition and nutritive value for different forms of DGc confirms the common view about their extreme variability [Nuez-Ortín et al., 2010, Olentine et al., 1986].
The main producer of corn distillers' grains (DGc) worldwide is USA, while EU and Canada used wheat as a raw material for bioethanol and corn is ranked second.
The aim of this study was to characterize and make a comparative analysis of the main subproducts of ethanol industry - DDGc / DDGSc.

Bioethanol industry represent a complex and multistage process, which generally can be illustrated in the following scheme:

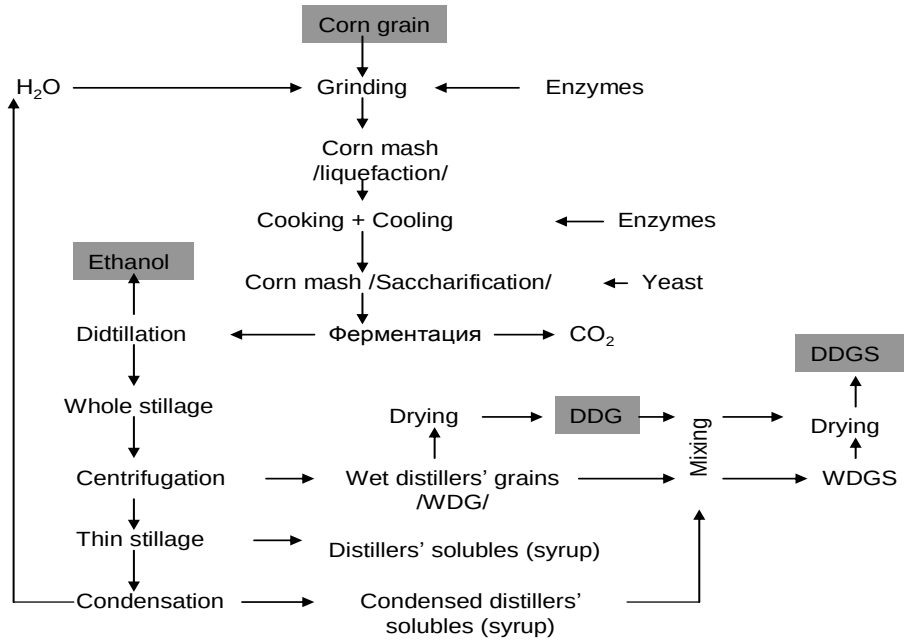


Figure 1. Schematic presentation of the manufacturing of DG products by dry method (www.trc.zootechnie.fr)

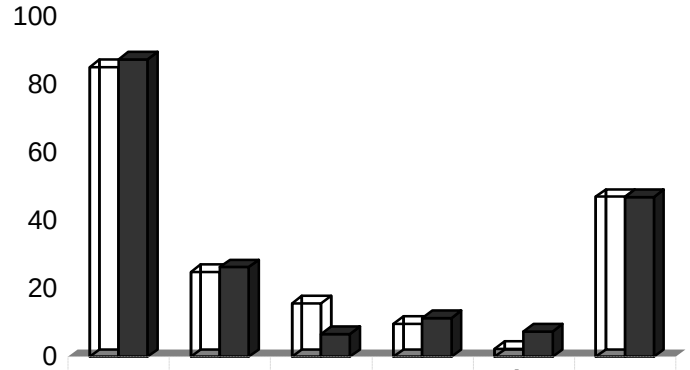
Our interest in this feed stems from the fact that it can be considered as a essential part of farm animals' rations. DDGSc is rich in proteins, moderately high in fats and relatively low in fibers, which allows to be used in the rations of different types and categories of farm animals [Hayes, 2008]. They can be added as both protein and energy source.

MATERIAL AND METHODS

Representative samples of DDGc and DDGSc were taken from the largest Bulgarian producers of bioethanol. Samples were stored in paper bags, and then - in glass bottles until laboratory analysed. The determination of dry matter (DM) was under AOAC [2002]. Total chemical analysis including following indices: dry matter (DM), crude protein (CP), crude fiber (CF), ether extact (EE) and ash (CA) according to [AOAS, 2002]. The amino acid profile and analysis of mineral substances have also been carried out according to AOAC [2002]. The results were analyzed using statistical package MS Office 2007.

RESULTS AND DISCUSSION

The results of the total chemical analysis are summarized and shown in Figure 2.
Data in the table above shows comparatively similar indices about DM, CP, and NFE, and variability in the other parameters of products - CF, Ash and EE.
DDDGc and DDGSc dry matter values were 85.19 and 87.42%, respectively. These relatively similar values are a good basis for comparison and interpretation of the chemical parameters, and their respective nutritive values.The protein content in both products is approximately the same - 25.03% in DDGc and 26.40% in DDGSc.



	DM, %	CP, %	CF, %	EE, %	Ash, %	NFE,%
DDGc	85,19	25,03	15,78	9,74	2,38	47,07
DDGSc	87,42	26,4	6,69	11,46	7,47	46,99

Abbreviations:
DDGc - dried distillers' corn grains;
DDGSc - dried distillers' corn grains with solubles;
DM – dry matter;
CP - crude protein;
CF – crude fiber,
EE – ether exract;
NFE – nitrogen-free extractives.
* Data based on our own analysis

Figure 2. Chemical analysis of DDGc and DDGSc* (DM basis) (n = 3 ± SD):

Our results for protein content are similar to those reported by Noblet et al., [1993], but lower than those found by Al-Suwaiegh et al., [2002]; Gibb et al., [2008]; Kessen et al., [2005]; Macken et al., [2004]; MAFF, [1990]; Moujahed et al., [2009]; Stalker et al., [2009]; Tjardes et al., [2002]; Uwituze et al., [2011]. Significantly lower levels of protein were reported by Carvalhoa et al., [2005]; [www.extension. astate. edu]; [Zarnela.pdf]. It is worth noting the difference in actual values obtained for fat content - 9.74 and 11.46%, respectively, in DDGc and DDGSc. Similar values were reported by Uwituze et al., [2011] and [Zarnela.pdf], unlike Kessen et al., [2005]; MAFF, [1990]; Noblet et al., [1993]; Stalker et al., [2009]; Tjardes et al., [2002], who found significantly lower values. Mosier et al., [2006] reported higher values.
It's worth to note the great difference in the actual value of the crude fiber. Almost a threefold advantage of DDGc (15.78%) to DDGSc (6.69%) for this index. The data correspond with those reported by Noblet et al., [1993], but are lower than those found by Moujahed et al., [2009]; Tjardes et al., [2002]; [Zarnela.pdf]. Ash quantity in DDGSc was several times higher (7.47%) comprelative for that in other research product - DDGc (2.38%). The calculated actual values for nitrogen-free extractives in both tested feeds are extremely close - 47.07 and 46.99%, respectively, for DDGc and DDGSc. These data are consistented with the fact that in the process of ethanol manufacturing starch is extracted from the raw material, while the rest of carbohydrates remains as so-called ''physiologically active fibers'' (Li et al. 2011).

	GE	OE	NEg	FUG	NEl	FUM	CP	PDI	BPR
DDGc	16,71	8,63	0,42	1,2	0,61	1,08	250,3	155	32
DDGSc	16,54	8,49	0,41	1,19	0,59	1,07	260,4	136,2	68,82

Abbreviations:
GE – gross energy,
DE – Digestible energy,
NEg – Nett energy for gain,
FUG – feed units for gain;
NEl – Nett energy for lactation,
FUM – feed units for milk,
CP– crude protein,
PDI– protein truly digestible in small intestines,
BPR– balance of protein in rumen.
* based on our own analysis

Figure 3. Nutritional value of DDGc and DDGSc * (in kg DM) (n = 3 ± SD):

Assessment of the nutritive value of both feeds was based on comparison of their energy and protein nutritive values. For that purpose were conducted series of differential balance experiments with the wethers reared at the experimental facilities of the Institute of Animal

Science Kostinbrod. The results are summarized and presented in Figure 3. Calculated values for energy and protein are consistent and logical consequence of the actual chemical composition. Energy nutritive value of both feeds, compared together, is presented in international units - NE, OE, NEl and NEg, as well as in forage units - FUG and FUM. The obtained values were significantly lower than those calculated by Gibb et al., [2008] and NRC [2007]. There was a trend towards slight superiority of DDGc over DDGSc. Values about FUG and FUM in DDGc and DDGSc were as follows - 1.20, 1.19, 1.08 and 1.07. They are significantly lower than those published by Tjardes et al., [2002], but similar with data obtained Crawshaw [2007] and MAFF [1990]. Lower values were obtained by Dimova et al., [2009].

The assessment of protein nutritive value was based on the following items: CP, PDI and BPR. Crude protein content in DDGc and DDGSc was 250.30 and 260.40g/kg DM, respectively. That's a contrast of 10.10g/kg DM. The calculated values for PDI in DDGc and DDGSc, based on conducted balance experiments [Yossifov et al., 2011] were as follows - 155.0 and 136.17g/kg DM. The lower values of PDI in DDGSc were due to differences in the technological processing and especially to the heat treatment. Therefore, higher temperatures probably led to denaturation of some protein components and fractions. Thus reducing DDGSc crude protein degradation, and the amount of PDI respectively.

Yet, BPR correlated positively with the amount of crude protein - (32.0) and (68.82) in DDGc and DDGSc, respectively. These data come to show that both feeds secured satisfactory supply of rumen microorganisms as evidenced by microbial protein synthesis. The values of BPR and PDI were lower than those established by Tjardes et al., [2002] and higher than those reported by Dimova et al., [2009].

The amino acid profile of both feeds is presented in Table 1:

Table 1. DDGc and DDGSc amino acid profile (n = 3 ± SD):

Value		% (g/100g feed)		% (g/100g CP)	
Feedstuff		DDGc	DDGSc	DDGc	DDGSc
Amino acid:	Aspartic acid,%	1.36	1.54	6.57	7.34
	Threonine,%	0.91	0.85	4.40	4.03
	Serine,%	0.77	1.27	3.75	6.04
	Glutamic acid,%	4.33	4.36	20.95	20.72
	Proline,%	2.12	2.38	10.26	11.34
	Cystine,%	0.58	0.40	2.83	1.89
	Glycine,%	0.84	0.69	4.06	3.26
	Alanine,%	1.29	1.76	6.25	8.39
	Valine,%	1.09	0.88	5.30	4.16
	Methionine,%	0.35	0.20	1.69	0.90
	Isoleucine,%	0.79	0.61	3.82	2.91
	Leucine,%	2.63	2.44	12.72	11.59
	Tyrosine,%	0.54	0.61	2.61	2.89
	Phenylalanine,%	1.02	1.05	4.92	5.01
	Histidine,%	0.69	0.64	3.33	3.06
	Lysine,%	0.55	0.69	2.67	3.29
	Arginine,%	0.80	0.73	3.88	3.48
Total, %		20.66	21.03	100.00	100.00

The quantity of individual amino acids (AA) was well balanced and apportioned according to the persentage of CP in the feeds included in the livestock diets. Individual AA can be combined into three groups, according to their quantitative distribution in the studied feed:

- AA which are relatively constant (variability within 10.0%) - threonine, glutamic acid, leucine, histidine, phenylalanine, arginine;
- AA which are relatively volatile (variability within 20.0%) - proline, aspartic acid, glycine, valine, tyrosine;
- AA which are highly variable (variability over 20.0%) - serine, cysteine, methionine, isoleucine, alanine, lysine.

Based on the analysis, it's worth to note the low percentages of AA lysine and methionine. This will inevitably reflect on the expected performance in both high-productive ruminants and monogastric species.

Regarding the AA differences between both feed, DDGc exceled DDGSc in the quantity of the following nine AA - threonine, cystine, glycine, valine, methionine, isoleucine, leucine, histidine and arginine. The quantity of the remaining eight AA (lysine, phenylalanine, tyrosine, alanine, proline, serine, glutamic and aspartic acid) as assessed in 100g sample was higher in DDGSc, relative to DDGc. Similar results were found regarding AA content in 100g crude protein. In this case DDGc was superior to DDGSc at 10 AA - threonine, glutamic acid, cystine, glycine, valine, methionine, isoleucine, leucine, histidine and arginine. The superiority of DDGSc was at 7 AA - aspartic acid, serine, proline, alanine, tyrosine, phenylalanine and lysine.

AA content in DDGc and DDGSc, calculated on DM basis, was 20.66 and 21.03%, respectively. As a percentage of protein, were 82.54 and 79.66% in DDGc and DDGSc, respectively. The remainder to 100% CP (so-called non-protein nitrogen (NPN)) was 17.46 and 20.34% in DDGc and DDGSc, respectively. The effect of theso-called Maylard reaction should be taken into account, when interpret the factual data in Table 3. Due to this reaction some of the AA (especially lysine) bind to physiological fibers toform insoluble compounds, mainly acrylamide, which further reduces the bioavailability of individual AA in the gastrointestinal tract.

Table 2 shows the content of minerals in the investigated feeds:

Table 2. Mineral composition of DDGc and DDGSc (n = 3 ± SD):

Item		DDGc	DDGSc
Menerals:	Ca^{+2}, %	0.19	0.44
	P^{+5}, %	0.44	1.21
	K^{+}, %	0.20	NA
	Na^{+}, %	0.24	NA
	Mg^{+2}, %	0.078	NA
	Fe^{+2}, ppm	218.0	86.41
	Zn^{+2}, ppm	39.0	59.73
	Mn^{+2}, ppm	19.0	21.21
	Cu^{+2}, ppm	21.0	6.80
	Ni^{+2}, ppm	NA	3.24
	Cr^{+2}, ppm	NA	0.35
	Pb^{+2}, ppm	-	-

Calcium and phosphorus are traditionally taken as a limiting factor in the utilization of by-products of ethanol industry by the animals and achievement of optimum productivity and environmental protection. The content of calcium kations in DDGSc, evaluated on DM basis, was twice lower of compared to that in DDGc, and amounted to 0.44 and 0.19%, respectively. The amount of phosphorus, however, showed even greater variability - 1.21 and 0.44%. The levels of calcium and phosphorus were close to those published by Tjardes et al., [2002], but lower than those found by Gibb et al., [2008] and Uwituze et al., [2011] and higher than thoses found by [Zarnela.pdf].

Potassium, sodium, copper and sulfur concentrations are of great importance, since these elements could inflict toxicosis, when their concentrations exceed phisiological range. Heavy metal concentrations in both feeds were within the admissable norms.

Based on a series of experiments, chemical analyzes and determination of several parameters (chemical composition, in vivo digestibility, energy and protein nutritive value) of DDGc and DDGSc were obtained factual data that can be used for full evaluation and grading of feed, subject of this study.

Our data, based on the conducted chemical analysis of DDGc and DDGSc, confirm the view reported by Nuez-Ortín et al., [2010], Olentine et al., [1986] and Petkova et al. ., [2011]., that these feeds show great variability with respect to each of the chemical item in its

composition. This view isn't consistent with the information reported by Schingoethe et al., [2009].

CONCLUSIONS

- Data obtained on the chemical composition of DDGc were: DM - 85.19%; CP - 250.30g/kg DM, EE - 90.74g/kg DM; CF - 157.80g/kg DM, NFE - 470.70g/kg DM, Ca^{+2} - 0.19g/kg DM and P^{+5} - 0.44g/kg DM, and DDGSc: DM - 87.42%; CP- 264.00g/kg DM, EE- 114.60g/kg DM, CF -66.90g/kg DM; NFE - 469.90g/kg DM, Ca^{+2} - 0.44g/kg DM and P^{+5} - 1.21g/kg DM. These data confirm the variability in composition of the investigated by-products of ethanol industry;
- Energy nutritive values expressed as FUM and FUG were 1.08 and 1.20 in DDGc, versus 1.07 and 1.19 in DDGSc;
- DDGc and DDGSc protein nutritive value was evaluated by CP (250.30 and 260.40g/kg DM), BPR (32.00 and 68.82) and PDI (155.00 and 136.17g/kg DM);
- Individual AA in the investigated feeds were well balanced and apportioned according to % of CP with clearcut pattern;
- The quantity of total AA in DDGc and DDGSc assessed on DM basis was 20.66 and 21.03%, respectively. As a percentage of CP, these values are 82.54 and 79.66%, respectively about DDGc and DDGSc. The remainder to 100% CP is so-called non-protein nitrogen (NPN). Their value in DDGc and DDGSc were 17.46 and 20.34%;
- Lysine and methionine content in 100g feed (DDGc / DDGSc) was as follows: 0.35 / 0.20 and 0.55 / 0.69g.

ACKNOWLEDGEMENTS

This paper is a result of a research within the project **«**Effective utilization of feed and reduction of environmental damage by feeding traditional, nontraditional, by-products from manufacturing process and feed additives**»**, financed by Agricultural Academy, Republic of Bulgaria.

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IMPROVEMENT OF TECHNOLOGY OF PRODUCTION OF THE MIXED FEED FOR CHICKENS

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ABSTRACT: The technologies of production of starting mixed feed don't consider the requirements of the poultry in homogeneous mixed feed with the high content of protein. The aim of the study was to increase the efficiency of production and use of mixed feed by extruding mixtures of the grinded grain and sub-standard eggs without shell.
Analitical and mathematic-statistical methods have been used in the work.
The technological way of enrichment of grain raw material by protein of an animal origin provides receiving of a preliminary mixture of the grinded grain of corn and sub-standard eggs without shell in the ratio 1:1, mixing of the preliminary mixture with the grinded corn which remained, and extruding received mixtures. The optimal quantity of eggs in the mixture is 10 %. The recipes of starting mixed feed have been developed on the basis of studying of requirements in nutritious and biologically active substances for chickens.
The improved basic technological scheme of manufacture of starting mixed feed for the poultry provides possibility of manufacture of the extruded feed additive (EFA) on the equipment of the feed mill. The necessity of receiving of preliminary mixture of microcomponents and extruded feed additive by two-phases mixing of them in the ratio 1:1 at the first stage of mixing and 1:2 at the second stage has been established experimentally. It is necessary for uniform distribution of microcomponents in mixed feed taking into consideration weight of single consumption of a forage by the poultry.
Key words: *poultry, mixed feed, technology, production, sub-standard eggs*

INTRODUCTION

Recently the tendency of increasing the requirements to quality of mixed feed, improving of technologies, cost cutting on production were observed in the mixed feed industry. In Ukraine the greatest share of the market is occupied by mixed feed for the poultry (47 %) which is the most exacting to quality and composition of forages (Yegorov, 2010). Producers try to use all possibilities for receiving the maximum profit with the minimum expenses, however it is necessary to support high quality of products for the market competitiveness.
Today out-of-date norms of feeding of the poultry are used. They don't meet the requirements of passports of up-to-date breeds, of chemical composition of raw material and of technology of production of the mixed feed. Recently the requirements in nutritious and biologically active substances for chickens have increased in connection with the use of high-productive breeds of the poultry that have high potential and are more sensitive to the quality and composition of feed (Taranu, 2000, Hunton, 1997, Gill, 2004, Brillard, 2001). At the same time the chemical composition of raw material for manufacture of mixed feed, first of all grain components, has been worsened considerably. The content of crude protein in wheat and corn has decreased on 1…2 absolute percent because of reduction in fertility of soils, intensive and wrong crop rotation, infringements of agrotechnology and unbringing organic and mineral fertilizers that makes difficult mixed feed balancing (Fushiki et al., 1985).
This problem can be solved by enrichment of grain raw material with animal protein which is assimilated easily. However, their use leads to growth of the cost of mixed feed. The peculiarities of digestion and high genetic potential of productivity of chickens cause necessity of using of high-quality mixed feed with the high content of the protein for its feeding, namely starting mixed feed.
In the first days of life daily giving of a forage is very small to the poultry, and furthermore single giving is. For chickens of egg lines daily giving is 7…8 g, and single – 0,88…1,33 g; for broilers – 15 g, and single – 1,88…2,5. All nutritious and biologically active substances provided with the recipe should be in each point of volume of a single portion of mixed feed for the poultry. Mixed feed should be homogeneous (Duncan, 1988, Behnke, 1994).

Everywhere used technologies of production of starting mixed feed for the poultry don't consider increased requirements to uniformity of distribution of nutritious and biologically active substances in the microvolumes. Their portions shouldn't exceed single consumption of mixed feed by the poultry. Consequently there was some need of searching of economically effective ways of increasing of fodder value of grain raw material and uniform distribution of nutritious and biologically active substances as a part of mixed feed.
The aim of the study was to increase the efficiency of production and use of mixed feed by extruding mixtures of the grinded grain and sub-standard eggs without shell.

MATERIAL AND METHODS

A complex of the standard analitical and mathematic-statistical methods of the research have been used in the work for studying of physical and chemical, biochemical and microbiological indexes of the extruded feed additive, starting mixed feeds and raw material.

RESULTS AND DISCUSSION

We offer a technological way of enrichment of corn by extruding of mixture of grains grinded to necessary size and eggs without shell, taking into consideration possibility of processing humidified grain to 16…18 % in grain extruders.
As a result of experimental researches it has been determined the necessity of mixing in two stages for obtaining of the high-quality homogeneous grinded corn enriched with egg protein. They are the obtaining of a preliminary mixture of components in the ratio 1:1 in the frame mixer during 180 sec and the basic mixing of the preliminary mixture of components and a part of the grinded corn which remained in the paddle mixer during 120-180 sec. The coefficient of variation of the received mixture is 2,7 %.
It has been determined experimentally that the optimal quantity of eggs without shell in the mixture with the grinded corn is 10 % under conditions of the minimum specific expenses of the electric power on manufacture and the best indexes of quality of EFA (Figure 1, 2).

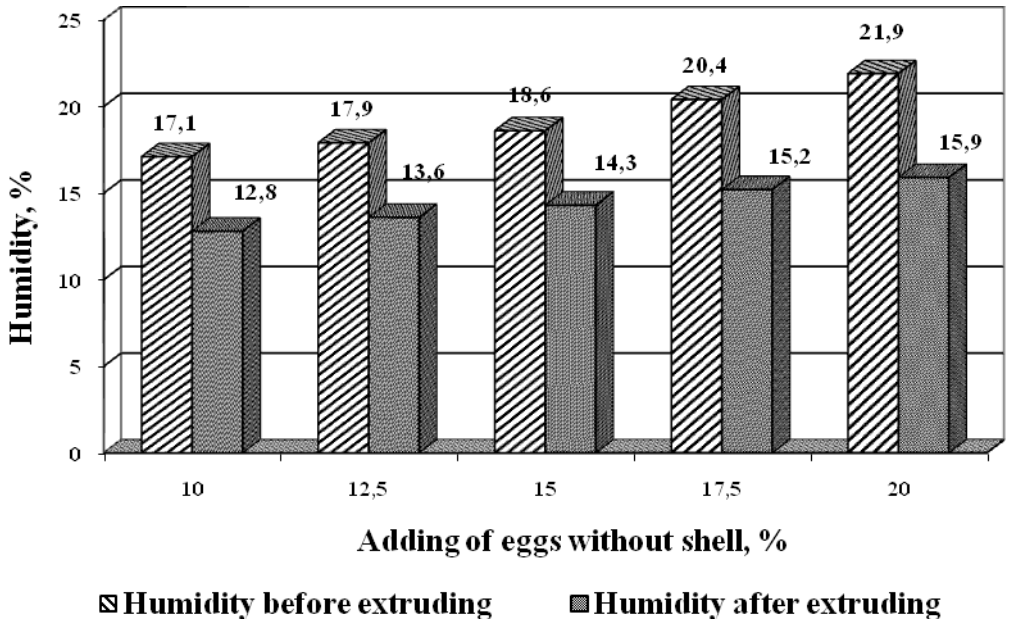

Figure 1. The changes of humidity in extruding depending on quantity of eggs in a mixture

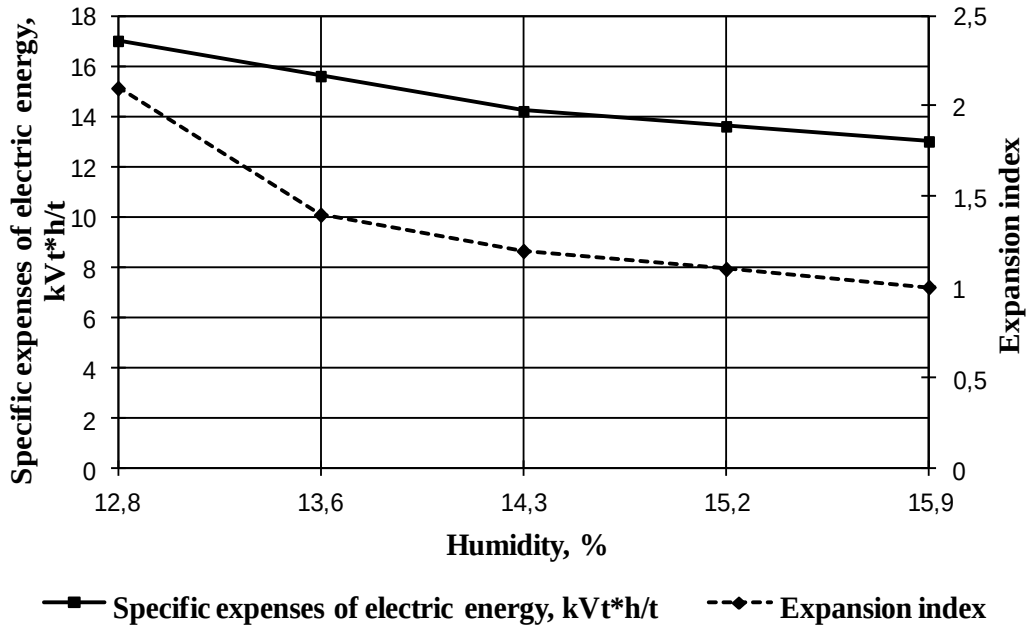

Figure 2. The dependence of specific expenses of the electric power and expansion index from humidity in EFA

On the basis of the experimental researches the basic technological scheme of manufacture of the EFA for the poultry and starting mixed feed have been developed (Figure 3). There are technological lines for manufacture of mixed feed:

1) of preparating of a portion of the clean and grinded grain raw material;
2) of preparating of a portion of mineral and mealy raw materials which don't need grinding;
3) of preparating of a portion of a preliminary mixture of microcomponents;
4) mixing of all prepared portions;
5) pelleting of mixed feed.

Adding of EFA is provided on the two technological lines simultaneously according to the improved technology (obtaining a portion of components which don't need any grinding, and a portion of microcomponents). As it was mentioned above, the single giving of starting mixed feed is very small and all nutritious and biologically active substances should be distributed uniform in it. The greatest problem is caused by distribution of microcomponents. Some of them contains in the recipe in quantity less than 0,1 %.
We have carried out the researches of the change of uniform distribution of microcomponents in samples depending on increase of their weight. It is offered to receive preliminary mixture of microcomponents and EFA by the way of their mixing in two stages in the ratio 1:1 at the first stage of mixing and 1:2 at the second stage (Figure 4). As we see, the less the weight of sample is, the more the mixture ununiform is. The coefficient of variation of preliminary mixture of microcomponents and EFA is 2,3 % after mixing in two stages in paddle mixer for 120-180 sec in sample weight of 0,5 g.
Production of EFA can be carried out on the equipment of the feed mill with adding of some equipment according to the developed basic technological scheme of manufacture of starting mixed feed for the poultry. This leads to considerable decreasing of capital investments on realization of improved technology of enrichment of grain raw material with animal protein.

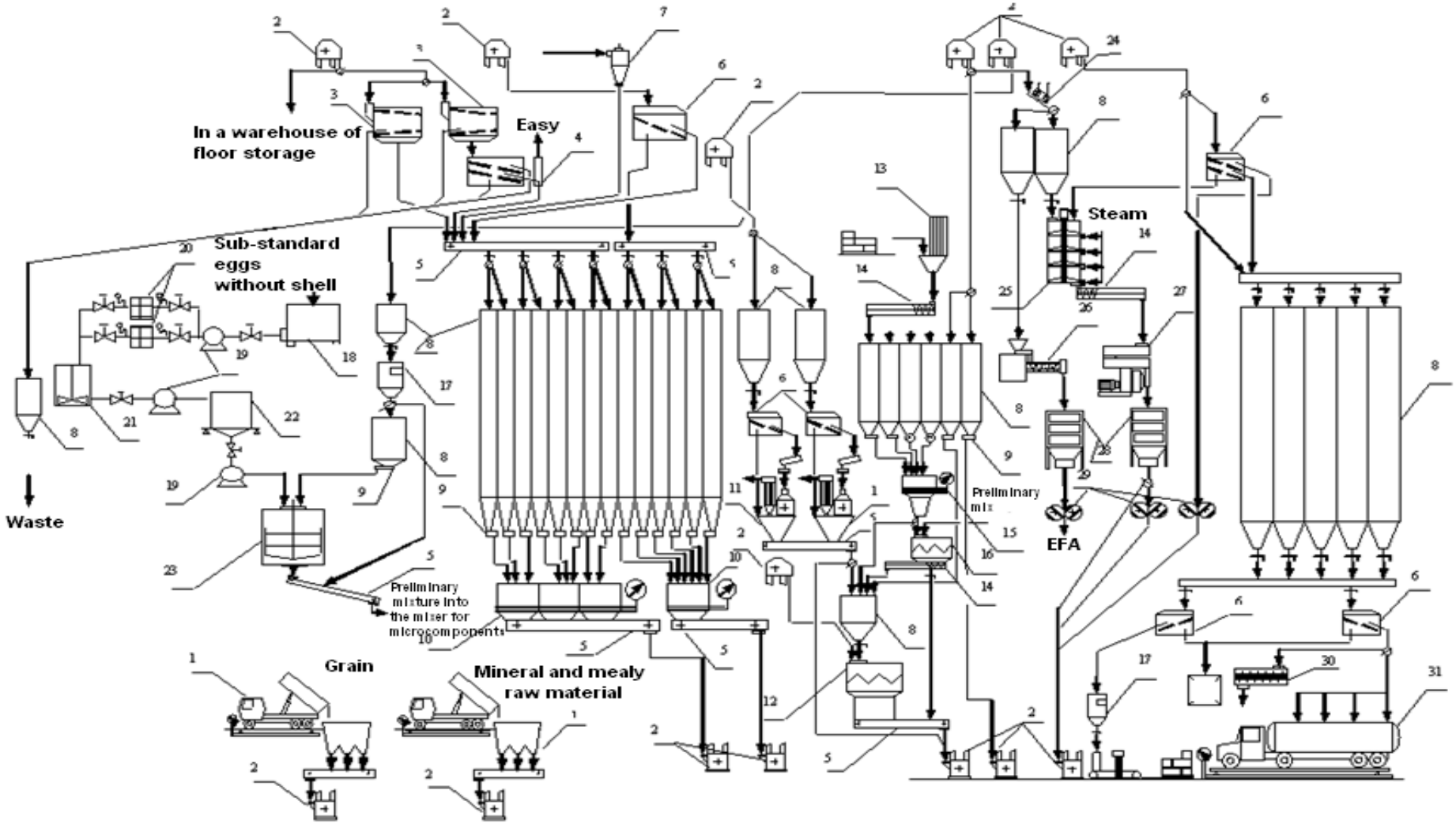

Figure. 3. The basic technological scheme of manufacture of starting mixed feed for the poultry:1–reception of raw materials, 2–elevators, 3–scalperator, 4–sieve/air separator, 5–transporter, 6–sifter 7–cyclone/discharger, 8–bunker, 9–screw conveyor, 10–multi component batch scale, 11–grinders, 12–mixer 13 and 14–screw conveyers, 15–multi component batch scale, 16–mixer, 17–scale, 18–container, 19–pump, 20–filter, 21–bunker with mixer, 22–bunker on gages, 23–frame mixer, 24–magnetic separator, 25–conditioner, 26–extruder, 27–pellet press, 28–cooler, 29–rolling grinder, 30–sprayer for liquids, 31–delivery of a finished product.

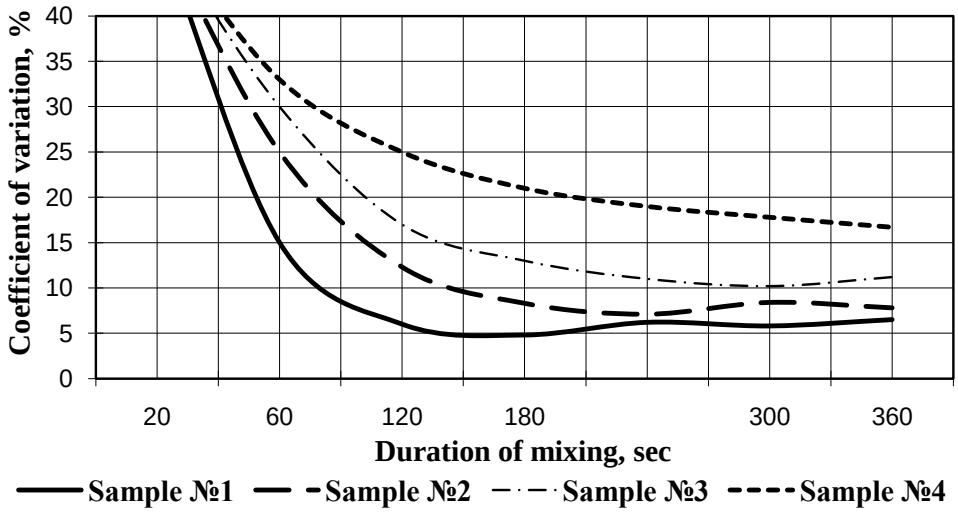

Figure 4. Estimation of uniformity depending on weight of sample:
1 – m = 2 g, 2 – m = 1,5 g, 3 – m = 1 g, 4 – m = 0,5 g

CONCLUSIONS

The technology ofproduction of mixed feed is improved on the basis of theoretical and experimental researches. It have been proved that the microvolume in which all components of mixed feed should be uniform distributed shouldn't exceed single consumption of mixed feed by the poultry.

The technological way of enrichment of grain raw material by protein of an animal origin provides receiving of a preliminary mixture of the grinded grain of corn and sub-standard eggs without shell in the ratio 1:1, mixing of the preliminary mixture with the grinded corn which remained, and extruding received mixtures. The improved basic technological scheme of manufacture of starting mixed feed for the poultry provides possibility of manufacture of the extruded feed additive (EFA) on the equipment of the feed mill. The necessity of receiving of preliminary mixture of microcomponents and extruded feed additive by two-phases mixing of them in the ratio 1:1 at the first stage of mixing and 1:2 at the second stage has been established experimentally. It is necessary for uniform distribution of microcomponents in mixed feed taking into consideration weight of single consumption of a forage by the poultry.

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VERTICAL COORDINATION IN POST-TRANSITION RED MEAT SUPPLY CHAIN: THE CASE OF SLOVENIA

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ABSTRACT: The key focus of the paper is to analyse the attitudes of pig and cattle breeders towards the collective action typical of contemporary supply chains. We investigated in detail the benefits or burdens from participation in a vertical chain. The data were collected in a survey including 313 farmers engaged in pig or cattle production. Respondents were asked to evaluate statements related to organizational forms of collective action and its benefits, express their attitude towards the supply chain coordination, evaluate currently established relationships, etc. The hierarchical cluster analysis was used to group the respondents regarding their agreement levels on different aspects of supply chain relationships. Other parts of the questionnaire were used to form and describe cluster profiles. The results have shown that obsolete and inefficient organizational forms of supply chain relationships prevail in Slovenian pig and cattle breeding sectors, however, farmers significantly differ according to their attitude towards vertical coordination. Moreover, alternative forms of collective activities are set up in order to overcome weakness and inefficiency of the prevalent vertical chains.
Key words: *red meat supply chain, vertical coordination, collective action, supply chain relationships, breeders attitude, cluster analysis*

INTRODUCTION

Vertical supply chain integration brings economic benefits to all chain participants, especially to smaller and weaker partners, assures market competitiveness, improves efficiency of production process and sale, and also improves product quality. Several studies of vertical co-operation on mature markets have proven the advantages of vertical integration. Therefore, effective collective action is the key approach to improve competitiveness, especially in post-transitional economies, i.e. economies in the final stage of adaptation to globalized market circumstances (Boger et al., 2001; Boger, 2001; Kuhar et al., 2010). Agriculture in Slovenia is part of this process as well. Slovenian agriculture is characterized by dispersed farm structure, which is beside lack of efficient supply chain operation an important factor for unfavourable economic performance. Among the subsectors of Slovenian agriculture, the red meat supply chain shows several symptoms of a sector in intensive transformation, yet many characteristics are annotating pre-transitional operation models (Kuhar et al., 2010). The unfinished transformation process of the sector dictates investments of additional efforts to conclude the transition period of *the formation of* economically efficient agriculture. For effective encouragement of collective activities it is necessary to be acquainted with breeders' aspect of supply chain relationships. The main focus of the paper is therefore to analyze the attitudes of pig and cattle breeders towards the collective action typical of contemporary supply chains and the importance that pig and cattle breeders attribute to a collective activity. Information about breeders' attitude towards co-operation is very important to formulate an effective strategy for stimulating quality collective activities (Kuhar et al., 2010).

To obtain information about breeders' attitude towards collective action the respondents were inquired to evaluate statements related to organizational forms of collective action and its benefits, such as higher product quality, prices and lower business risks, to express their attitude towards the supply chain coordination, to evaluate currently established relationships, etc. Clusters of breeders with a similar attitude towards co-operation were

formatted regarding the agreement levels on different aspect of supply chain relationship. - Furthermore, we described cluster profiles with socio-demographic breeders' characteristics such as age, level of education, employment status of farmer, number of animals fattened for sale and planned farm extent changes. Cluster members' profiles are also explained through most frequently used marketing channels and evaluation of their economical efficiency, main buyer category and buyer to whom most fattened animals have been sold. Information about a mode of co-operation with the main buyer and the evaluation of consideration of the eventual pre-contract were also used for cluster profiles formation.
In this paper we will present our research in three following sections. In the first section, we will explain research procedure and methods used to generate results demonstrated in the second section. First, we will present cluster characteristics (for cluster formation we have applied a hierarchical cluster analysis), and second, we will describe defined cluster profiles using fundamental descriptive statistics. In the final section we will report research conclusions and indicate abilities of applicative use.

MATERIAL AND METHODS

Data were collected between November 2009 and March 2010 through a postal survey of Slovenian cattle and pig breeders. The sample included 1000 cattle and 700 pig breeders enrolled in a central register of breeders at the Ministry of Agriculture. The response rate was 18 %, which means that the questionnaire was answered in by 313 respondents. Breeders were asked about relationships in an agrifood supply chain, cooperation modes, prevalent and main buyers. Survey data were analysed by a multivariate statistical technique and fundamental descriptive statistics. Respondents were first classified in three clusters according to their evaluation of statements related to organizational forms of collective action and its benefits such as higher product quality, prices and lower business risks, and according to their attitude towards the supply chain coordination (Boger et al., 2001). A hierarchical cluster technique – Ward's method was used for classification. Dissimilarity of standardized variables was measured by Euclidean distance. After close examination, the dendrogram three-cluster solution was considered the most appropriate (Ferligoj, 1989). Later, we described each of the three cluster profiles according to breeders' socio-demographic characteristics, most frequently used marketing channels, modes of cooperation and relationships with buyer. Cattle and pig breeders differ in marketing and also in cooperation modes, which we had already taken into consideration when formulating questions, and later, when formulating cluster profiles. The fundamental descriptive statistics were used for cluster profiles formulation.

RESULTS AND DISCUSSION

Differences among animal breeders about their attitude towards collective activities and their beliefs about benefits of cooperation enabled us to classify them in three different clusters. The first cluster, named *traditional co-operative breeders,* includes members who prefer cooperation and are aware of its benefits. According to their opinion, agricultural co-operative (a traditional form of agricultural co-operative that has existed on the Slovenian territory for nearly a century) is still the best agent between farmers and food industry. Traditional co-operative breeders represent the majority (45.6 %) of all respondents. The second cluster, *non-co-operative breeders,* represents 18.6 %, that is the least of all respondents. Non-co-operative breeders are not in favour of collective action. They disagree that collective action provides better prices, reduces business risk and expenses, and improves meat and meat product quality. The third cluster is characterized by progressive breeders, who have a positive attitude towards collective action, but a negative attitude towards cooperation in traditional agricultural co-operatives. Progressive breeders represent 35.6 % of the respondents.

Table 3. Clusters with a similar attitude towards collective activities (N = 239)

Statement (1 – totally disagree, 5 – totally agree)	**1** n=109	**2** n=45	**3** n=85	**Total mean**
If breeders co-operated fully, their economic situation would improve.	4,35	3,07	4,47	**4,15**
Agricultural co-operation is a first-rate agent between the farmer and food industry.	3,95	2,18	1,82	**2,86**
Agricultural co-operative service helps farmers to reach a better economic situation.	3,88	1,62	1,79	**2,71**
Agricultural co-operatives should sell food factories they own.	2,22	2,47	2,80	**2,47**
Profit among farmers, co-operatives, food industry and traders are fairly distributed.	1,83	1,22	1,15	**1,47**
Farmer and food processor co-operation brings about better meat and meat products' quality.	3,32	1,89	3,06	**2,96**
Contract with the buyer reduces farmers' business risk.	3,83	1,87	4,06	**3,54**
Contract with the buyer assures a better price than the sale on the open market.	2,75	1,42	2,41	**2,38**
Long-term co-operation with the buyer helps farmers' business economics.	3,90	2,18	4,00	**3,61**

Traditional co-operative breeders are breeders whose socio-demographic characteristics are closest to the characteristics of an average Slovenian animal breeder. The majority is between 40 and 60 years old (63 %) and almost 2/3 of them have high school education, while one third have elementary school education only. Among cattle breeders there is a major part of small breeders, the majority of pig breeders own large pig farms. In the first cluster, the highest (23 %) percent (above average (10.6 %)), is the one of cattle breeders that do not sell fattened animals. On average, 62 % of all breeders are not planning any changes in breeding extent in the following year, the next largest part are in favour of decreasing breeding extent. Traditional co-operative breeders act in the same way. Most frequently used marketing channels among first cluster cattle breeders are agricultural co-operatives or a permanent buyer without any pre-contract. The vast majority of members (85 %) do not avail themselves of contractual breeding or sale to a random buyer. In 52.5 %, most animals are sold through agricultural co-operation, but only 44 % of respondents consider this way the most economically efficient. Sale to a permanent buyer without a pre-contract is the most economically efficient in the opinion of 41 % of respondents, and 39 % of breeders sell most cattle through this channel. Pig breeders' marketing channels are more heterogeneous than cattle breeders'. The prevalent marketing channel is contractual breeding for a large pig farm, however, it is also frequently used to supply very small permanent and random buyers with pigs, usually to stick them at home. This channel cannot apply to cattle breeders, because cattle slaughter at home is illegal in Slovenia. Most fattened pigs are sold through contractual breeding by 46 % of pig breeders, but only 22 % of them value it as economically most efficient. On the other hand, 51 % of farmers think that sale to a permanent buyer is the most economically efficient, but only 39 % sell most animals through this channel. First cluster pig breeders often ascribe economical efficiency to the sale to a random buyer, but do not practice it a lot. According to their belief that agricultural cooperative is a first-rate agent between farmers and agro-industry, traditional co-operative breeders sell most of their animals through agricultural co-operatives. Agricultural co-operative is the main buyer for 78 % of cattle breeders and 37 % of pig breeders. For other 34 % of pig breeders, the main buyer is a large Slovenian processor.

40 % of farmers sell most pigs through contractual breeding. A greater part of traditional co-operative cattle breeders most often co-operate with the main buyer (60 %) without contract or through agricultural co-operative (32 %)Traditional co-operative pig breeders most often practice contractual breeding, agricultural co-operation or a precise oral agreement. More than 30 % of pig breeders think that pre-contract is fully considered and 50 % that it is considered in major part.

Table 2. Attributes determinating cluster profiles

ATTRIBUTES OF CO-OPERATION	TRADITIONAL CO-OPERATIVE BREEDERS (n=109)		NON-CO-OPERATIVE BREEDERS (n=45)		PROACTIVE BREEDERS (n=85)	
	Cattle breeders (57 %)	**Pig breeders (43 %)**	**Cattle breeders (49 %)**	**Pig breeders (51 %)**	**Cattle breeders (33 %)**	**Pig breeders (67 %)**
Please select mode of sale for fattened animals. (1 - never, 5 – exclusively)	Co-operative (3.6) Permanent buyer without pre-contract (2.9)	Contractual breeding for farm (2.6) Small buyer without pre-contract (2.6) Small random buyer (2.3)	Permanent buyer without pre-contract (3.3) Co-operative (3.0)	Small (3.1) and medium (2.6) permanent buyer without pre-contract Small (2.8) and medium (2.1) random buyer Large permanent buyer (2.3)	Permanent buyer without pre-contract (3.8) Co-operative (2.6)	Contractual breeding for farm (2.4) Small (2,8) and medium (2.2) random buyer Small permanent buyer without pre-contract (2.1)
In which of listed modes did you sell most of your fattened animals in the past three years?	Co-operative (52. 5 %) Permanent buyer without pre-contract (39. 3 %)	Contractual breeding (46. 2 %) Permanent buyer without pre-contract (38. 5 %)	Co-operative (40. 9 %) Permanent buyer without pre-contract (40. 9 %)	Permanent buyer without pre-contract (52. 4 %) Contractual breeding (28. 6 %)	Permanent buyer (66. 7 %) Co-operative (29.6 %)	Permanent buyer without pre-contract (47. 2 %) Contractual breeding (32. 1 %)
Which of listed modes is in your experience the most economically efficient?	Co-operative (44.1 %) Permanent buyer without pre-contract (40. 7 %)	Permanent buyer without pre-contract (51. 4 %) Random buyer (27 %)	Permanent buyer without pre-contract (42. 9%) Co-operative (38. 1 %)	Permanent buyer without pre-contract (47. 6 %) Random buyer (28. 6 %)	Permanent buyer without pre-contract (68. 0%)	Permanent buyer without pre-contract (45. 3 %) Contractual breeding (28. 3 %)
In which mode do you co-operate with your main buyer?	Without pre-contract (60. 0 %) Co-operative (31. 7 %)	Contractual breeding (34. 1 %) Co-operative (22. 0 %) Precise oral agreement (17. 1 %)	Without contract (72. 7 %) Co-operative (18. 2 %)	Without any agreement (45. 0 %) Contractual breeding (15. 0 %) Co-operative (15. 0 %) Precise oral agreement (15. 0 %)	Without contract (88. 9 %)	Without any agreement (33. 3 %) Contractual breeding (27. 5 %) Precise oral agreement (17. 6 %)
Experience with compliance with the contract	No contract (61 %)	High compliance (52 %) Full compliance (32 %)	No contract (76 %)	High compliance (33 %) Full compliance (33 %)	No contract (89 %)	Full compliance (59 %) Other options 13 % each
How often in the past three years did you sell fattened animals to listed buyers? (1 - never, 5 – exclusively)	Co-operative (3.8) Local trader (2.7)	Co-operative (2.9) Large processor (2.5)	Co-operative (3.3) Local trader (2.9)	Large processor (2.1) Co-operative (1.9) Small processor (1.7)	Co-operative (2.6) Local trader (2.2) Small processor (2.1)	Large processor (2.4) Co-operative (2.0) Medium-sized processor (1.7)
To which buyer did you sell most fattened animal in the past three years?	Co-operative (81. 4 %)	Co-operative (42. 9 %) Contractual breeding (40. 0 %)	Co-operative (71. 4 %)	Contractual breeding (25.0 %) Co-operative (18. 8 %) Local trader (18. 8 %) Large , medium-sized and small processor (12. 5 % each)	Co-operative (50. 0 %) Local trader (30. 0 %)	Contractual breeding (39. 5 %) Co-operative (28. 9 %)
In which category of buyers is your main buyer placed?	Co-operative (78. 3 %)	Co-operative (36. 6 %) Large processor (34. 1 %)	Co-operative (68. 2 %) Medium-sized processor (13. 6 %)	Surrounding households (23.8 %) Distant household (14. 3 %) Large processor (14. 3 %) Small processor (14. 3 %) Co-operative (14. 3 %)	Co-operative (37. 0 %) Local trader (22. 2 %) Small processor (22. 2 %)	Surrounding households (26. 4 %) Large processor (24. 5 %) Distant household (15. 1 %) Co-operative (15. 1 %)

Non-co-operative breeders could be further subdivided into three sub-clusters– part-time breeders that do farm besides a full-time job and do not live of farming income. Among second cluster members, there is a higher percentage of respondents who work on farm and are employed. In the second sub-cluster, there are breeders that will gradually abandon farming. 29 % of cattle breeders and 22 % of pig breeders stated that they would reduce animal breeding production. In the third sub-cluster, there are larger, self-sufficient breeders who can ensure their farm's economic success independently. Non-co-operative breeders' marketing channels do not differ from traditional co-operative breeders. The most frequently used channels among cattle breeders are still agricultural co-operative (41 %) and the sale to a permanent buyer without contract (41 %). Pig breeders most frequently sell fattened pigs to small permanent or random buyers. Sale to a small buyer is economically most efficient for almost 80 % of all respondents. The most frequent mode of fattened animals sale among pig breeders is in 29 % contractual breeding, for 24 % it is even the most economically efficient one. The main buyer for cattle breeders is in 68 % agricultural co-operative, through which 71 % of cattle breeders sell most animals, and in 14 % medium-sized processor. Pig breeders sell fattened animals to many different categories of buyers, mostly to surrounding households (24 %), distant households, large and small processors and co-operatives (14 %). Most animals are sold through contractual breeding or agricultural co-operative by 25 % of pig breeders. Non-co-operative breeders co-operate with the main buyer without pre-contract (73 % of cattle breeders) or without any oral agreement (45 % of pig breeders), which is expected behaviour according to their attitude to contracting (Table 1.).

Progressive breeders are representatives of active, flexible farmers, seeking new, favourable marketing channels. The percentage of younger breeders is above average, for 62.5 % farming is the only employment. Both, cattle and pig breeders declare for increasing production – the former in 18.5 %, the latter even in 21.4 %. Cattle marketing channels are similar to other clusters: sale to a permanent buyer without pre-contract or agricultural co-operative among cattle breeders and sale to a small permanent buyer, contractual breeding or sale to a small random buyer among pig breeders. 67 % of breeders that are in favour of new modes of cooperation sell fattened animals to a permanent buyer most frequently and 67 % of them find this mode the most economically efficient. The great majority still co-operates with the main buyer without any pre-contract. Pig breeders (47 %) most frequently sell animals to a permanent buyer without pre-contract, which is for 45 % of breeders the most economically efficient, or through contractual breeding (32 %), the most economically efficient mode for 28 % of breeders. The rest evaluate sale to a random buyer as the most economically efficient, this mode of sale is most frequently used for 21 % of pig breeders. Progressive breeders practice contractual breeding (27.5 %)in major part, one third co-operate with the main buyer without any oral agreement and 17 % on the basis of a precise oral agreement. 60 % of pig breeders have positive experiences with contract compliance. Cattle breeders' main buyers are in 37 % agricultural co-operative, 50 % sell most animals to them, in 22 % the main buyer is a Slovenian trader and small processor. Pig breeders sell most animals through contractual breeding or agricultural cooperative. The main buyer for 26.4 % of breeders is in the category of surrounding households, for 24.5 % large processor and for 30.1 % distant households and agricultural co-operative.

CONCLUSION

As we predicted breeders have different attitudes towards collective action. Majority of them is in favour of cooperation since they recognise its benefits. But there still exists a minor group of breeders who do not want to co-operate for a variety of reasons. Differences among breeders that want to act collectively mostly show inclination for organisational form. Therefore, traditional co-operative breeders cooperate with agricultural co-operative to a higher degree but the question is if they co-operate because of an actual economic efficiency that is often criticized, or because of breeders' inertness and disability to change activities. Traditional agricultural co-operative has played an important role in Slovenian agriculture since the beginning of the 20th century, and before reestablishment of the capitalistic market

organisation it was the only mode of collaboration, but during the transition was not able to adapt to changed market conditions and consequently lag behind in economic efficiency (Avsec & Nose, 2001). Cattle breeders remain loyal to traditional agricultural co-operation because they still operate in relatively favourable market environment where reduction in meat price and feed price rise do not force them to rationalize the production process and where the cut-out of income compensate by subsidy direct payment. On the other hand, global pig meat sector recession requires better, more efficient organisation of breeders (Kuhar et al., 2010). For this reason pig breeders prefer progressive, market-oriented collective action forms and operate this way as well. Heterogeneous marketing channels indicate the direction of searching for the most advantageous sale mode. Analysis of marketing channels and main buyer types shows that a theoretical attitude towards collective action is in reality in accordance with breeders' activity. In this way, traditional co-operative breeders mostly co-operate with agricultural co-operation and evaluate it as the most economically efficient, progressive breeders, however, prefer other modes of collaboration but not practice them. Sale to a small permanent or random buyer or to households leads us up to opportunistic behaviour of breeders. Opportunistic behaviour is in our opinion an expression of lack of understanding principles of collective acting and awareness of vertical chain benefits. Understanding of vertical coordination and awareness of vertical chain benefits are of great importance for improvement of sector conditions. Therefore, it would be reasonable to pay more attention to this topic, especially by state institutions.

ACKNOWLEDGEMENTS

This research is part of project CRP V4-040 "Oskrbne verige rdečega mesa v Sloveniji – analiza stanja in oblikovanje razvojnih modelov za doseganje konkurenčnosti (Read meat supply chains in Slovenia background analysis and development of models for boosting competitiveness)", financed by Slovenian Research Agency and Ministry of Agriculture, Forestry and Food of Republic of Slovenia.

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USEFULNESS OF UREA AND PROTEIN CONCENTRATIONS IN MILK AND BLOOD AS INDICATORS OF NITROGEN UTILIZATION IN THE SIMMENTAL AND HOLSTEIN-FRIESIAN LACTATING DAIRY COWS

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ABSTRACT: The aim of this study was to determine the protein status in Simmental and Holstein-Friesian cows, as well as use possibilities of milk and blood urea values as indicator of nitrogen utilization. The digested N is mainly absorbed as free amino acids and is used by the animals for protein synthesis, or as ammonia, which needs to be detoxified by the liver into urea. The forms in which N is absorbed are critical in determining the proportion of digested N that is available to support protein synthesis. Investigation has been conducted on 17 lactating Simmental cows and 10 Holstein-Friesian lactating dairy cows divided into groups regarding to milk production (under 15 kg (S<15; H<15) and over 15 kg (S>15; H>15) and to lactating stages (under 100 days S<100d; H<100d and over 100 days). Blood pH and electrolytes (Na^+, K^+, Cl^-) have been determined by the RapidLab 348 (Bayer, Germany) analyzer. Blood metabolites (BUN, TP and albumin) have been measured by Beckman AU400 automatic analyzer. MUN (milk urea nitrogen), protein and fat content have been determined by infrared spectrophotometric method on Combi-Foss analyzer FT 6000.
Simmental cows (S<100d) had the lowest BUN and significantly lower MUN concentrations and lower MUN has been noticed also in H<15kg. Significantly higher TP concentration was noticed in H<15kg group and H>100d due to raised globulin count, probably because of inflammation. H>15kg had the lowest rumen pH, but blood pH was in referent interval in all groups. There was no correlation between BUN and MUN (r=0.32 in S>15L) which is related to the ratio of dietary CP to energy, because of excess of microbial requirements.
In the condition of low milk production as a consequence of low dietary protein and energy, MUN concentration is not relevant enough as an indicator of protein/energy supply.
Key words: *milk urea concentration, dairy cows, low protein diet*

INTRODUCTION

It is really important to establish good dairy farm management based on proper cows' nutrition because diet cost gets around 40 to 60% of the total milk production cost. Milk urea nitrogen (MUN) and milk protein are used as indicators of the protein-energy balance and for monitoring feeding disorders. Rumen degradable protein (RDP) is used by rumen microorganisms to synthesize microbial proteins, so ammonia has a great role in symbiosis maintenance between rumen micro flora and cow (Huntington and Archibeque, 1999). There are different reports dealing with the factors which influence MUN values: BUN, the effect of parity, time of lactation, milk yield, energy and protein metabolism in the rumen (Carlsson et al., 1995; Eicher et al. 1999, Arunvipas et al., 2003). During protein fermentation amino acids have been deaminated and ammonia occurs which is used by microorganisms for their own proteosynthesisIn case of insufficient energy supply more toxic ammonia accumulate, diffuse into ruminal epithelium and enter circulation. In the liver toxic ammonia is being converted into non-toxic urea. Urea is a hydro soluble molecule which can be found in blood, milk, urine and saliva. It is synthesized in the liver from ammonium obtained as a product of decomposition of food protein. Urea goes along to the kidneys and saliva glands, after that in saliva in the mouth and in the rumen again. Ammonia is absorbed across all sections of the

digestive tract of ruminants. Before diffusion through the gut epithelium the ammonium ion is converted to ammonia, diffuses into epithelial cells as ammonia, then protonated to re-form an ammonium ion. There are mechanisms providing transport of the ammonium ion, such as association with bicarbonate or VFA (volatile fatty acids) anions (Parker et al. 1995), but urea recycling may not only play a role in supplying nitrogen for microbial growth, but also in the maintenance of ruminal pH (Zhonyan et al., 2011). Milk protein consists of approximately 95% true protein and 5% non-protein nitrogen. About half of that non-protein fraction is urea, depending on the protein and energy supply (Geerts et al., 2003). The importance of MUN and BUN control is seen as follows: high amount of dietary protein had negative effect on reproduction (Hammon et al., 2005) and milk yield. High protein intake requires more energy to convert NH_3 to urea in the liver and releases more N into the atmosphere contributing to environmental pollution (Reynal and Broderick, 2005). According to Kauffman and St-Pierre (2001) there is a strong correlation (0.88 – 0.98) between MUN and BUN, therefore it is cheaper to use MUN as efficient parameter for N utilization in cows. Jonker et al. (1998) emphasised that MUN values for cows that were fed according to the National Research Council recommendation were from 10 to16 mg/dl depending on milk production.
The aim of this study was to examine the usefulness of BUN and MUN concentration under low diet protein condition for the assessment of nitrogen utilization.

MATERIAL AND METHODS

The trial was carried out on two dairy farms, one Simmental and one Holstein lactating dairy cows, average 2,4 parities divided recording to milk production (under 15 kg (S<15; H<15) and over 15 kg (S>15; H>15) and regarding to lactating stages (under 100 days S<100d; H<100d and over 100 days S>100d, H>100d). Blood for the biochemical parameters and acid base status was collected after morning milking, two hours after feeding from v. caudalis into sterile vacuum tubes. The blood pH and electrolytes (Na^+, K^+, $HCO3^-$, Cl^-) have been determined by the RapidLab 348 (Bayer, Germany) analyser, on the ion selective electrode principle. Blood urea nitrogen (BUN), total protein, albumin, Mg, Ca, P were assigned by Beckman Coulter AU 400 automatic analyser. MUN (milk urea nitrogen), protein and fat content have been determined by infrared spectrophotometric method on Combi-Foss analyzer FT 6000. The statistical analyses were performed by using the general linear model procedure of the StatSoft, Inc. Statistica (2008).

RESULTS AND DISCUSSION

Dairy cows were fed with TMR which nutritional composition is shown in the Table 1.

Table 1. Chemical analysis of TMR for dairy cows Simmental and Holstein breed

Ingredient, % of DM	Simmental	Holstein
DM	50.03	69.8
CP	11.4	12.68
Fat	1.9	2.7
Crude fibre	24.29	18.5
Ash	6.84	6.24
NEL MJ/kg DM	6.59	6.59

The mineral concentration was in referent interval in both breeds except chloride concentration which was low in Holstein cows. Low BUN concentration was in Simmental cows with lower milk yield. Total protein (TP) concentration was significantly lower in S<15 in relation to H<15, but albumin concentration was normal, probably due to higher globulin concentration. This is in accordance with Šamanc et al. (2011) who found low TP and

albumin concentration in cows with fatty liver. Cows of both breeds had very low milk protein (S<100d; H<100d).

Table 2. Blood minerals, metabolite concentration and milk compound in dairy cows according to days of lactation

	S<100d	S>100d	H<100d	H>100d	Breed x DIM P-value
Na, mmol/L	142.5^{a}±1	144.36^{a}±1.29	142.6^{a}±2.07	139.33^{b}±1.03	0.0002
K, mmol/L	4.52±0.53	4.27±0.2	4.33±0.22	4.39±0.78	0.4104
Cl, mmol/L	95.5±2.08	97.73±1.95	94±2.35	91.67±4.18	0.0572
Mg, mmol/L	0.89±0.05	0.95±0.11	0.82±0.13	0.88±0.1	0.9894
Ca, mmol/L	2.27±0.08	2.2±0.2	2.14±0.19	2.23±0.09	0.2673
P, mmol/L	1.99±0.39	2.19±0.42	1.56±0.39	1.37±0.5	0.2905
BUN, mmol/L	3.01±0.25	3.61±0.59	3.8±0.84	3.87±1.27	0.4462
TP, g/L	62.58±7.63	70.35±4.53	74.5±6.01	82.87±6.34	0.9028
Albumin, g/L	27.58±4.51	31.27±1.92	30.3±1.67	29.88±2.4	0.0635
Milk protein, %	3.14±0.57	3.92±0.81	3.3±0.39	3.48±0.55	0.3074
MUN, mg/100ml	11.75^{a}±2.87	16.82^{b}±4.85	15.8^{ab}±5.81	12.8^{ab}±2.49	0.0484

DIM-days post-partum
^{a,b} *different superscript differ significantly (P<0.05)*

MUN was significantly (P<0.05) lower in S<100d. Regarding the milk yield, the lowest BUN concentration had cows with low production (S<15), as well as TP concentration in relation to the Holstein cows. Cows H<15 had MUN concentration under referent limit (Table 3).

Table 3. Blood minerals, metabolite concentration and milk compound in dairy cows according to milk yield

	S<15	S>15	H<15	H>15	Breed x Milk yield P-value
Na, mmol/L	142.75±0.96	144.27±1.42	139.83±0.98	143±2.16	0.1948
K, mmol/L	4.49±0.53	4.28±0.21	4.42±0.77	4.33±0.25	0.7726
Cl, mmol/L	97.5±1.73	97±2.37	91.5±4.23	95±0.82	0.1071
Mg, mmol/L	0.92±0.11	0.94±0.1	0.84±0.15	0.87±0.04	0.9341
Ca, mmol/L	2.26±0.1	2.21±0.2	2.29±0.1	2.07±0.11	0.2385
P, mmol/L	2.1±0.38	2.15±0.43	1.44±0.47	1.59±0.45	0.8016
BUN, mmol/L	3.18±0.16	3.55±0.65	3.82±1.31	4.06±0.68	0.8593
TP, g/L	66.75^{a}±5.58	68.83^{a}±6.71	81.98^{b}±5.41	71.9^{a}±1.75	0.0247
Albumin, g/L	28.83±5.34	30.82±2.02	29.93±2.35	30.9±1.15	0.6714
Milk protein, %	3.46±0.84	3.81±0.82	3.41±0.53	3.38±0.41	0.5513
MUN, mg/100ml	15.25±2.36	15.55±5.65	12.83±2.23	16.5±6.45	0.4294

^{a,b} *different superscript differ significantly (P<0.05)*

It is known that decreasing RDP (rumen degradable protein) intake results in linear decreasing of milk true protein content, MUN, and blood urea-N (Reynal et Broderick, 2005). Castillo et al. (2001) studied the effect of supplementing grass silage diet with isoenergetic concentrates containing 2 levels of protein concentration at 3 levels of protein degradability on N use and found that milk yield (mean 23.8 kg/d), milk composition, and faecal N excretion were not affected by protein concentration or degradability. The main route for dietary N output in excess of requirements was via the urine (Broderick, 2003).

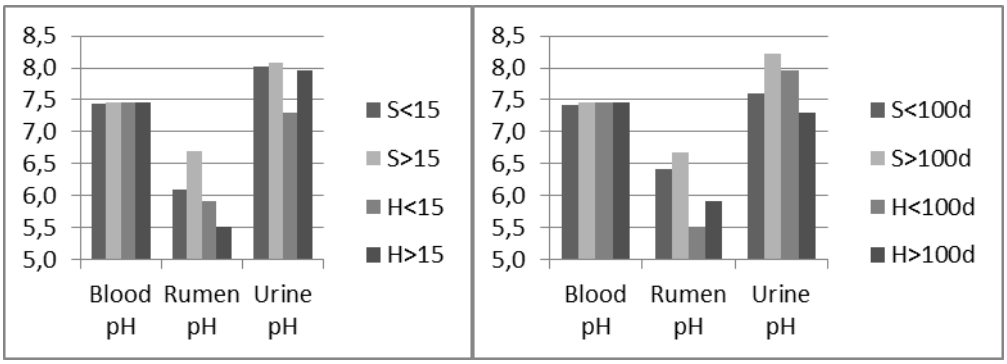

Figure 1. Blood, urine and rumen pH values in dairy cows according to milk yield and days of lactation

There is some speculation that feeding with RDP below the requirements for maximal rumen microbial growth can compromise microbial protein production, ruminal digestion, and energy and protein availability to the cow. In our study there was no significant correlation between BUN and MUN, but there was significant positive correlation between blood pH and total protein (r=0.74) in S>15 and S>100d (r=0.64) as well as blood protein and MUN (r=0.62). Following severe reduction in the protein content of the diet, a decreasing of BUN concentration is expected (Campanile et al, 1998).
As it is shown in Figure 1, blood pH was in the narrow lines, but rumen pH was the lowest in H<100d and H>15. Urine pH was significantly lower in H>100d and H<15. The rumen pH values were in referent range (except H<100d and H>15), which indicates that digestive processes run optimally.

CONCLUSION

Except nutritional factors, there are many non-nutritional factors which influence milk urea nitrogen levels in dairy cows. In the condition of low milk production due to low dietary protein and energy intake, MUN concentration is not relevant enough as an indicator of protein/energy supply. We found low BUN and MUN concentration, but weak coefficient correlation shows that nutritional processes are not rational and other metabolic pathways are included.

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SUCCESSFUL COMPLETE SUBSTITUTION OF FISH MEAL WITH PLANT PROTEIN INGREDIENTS IN DIETS FOR COMMON CARP, *CYPRINUS CARPIO L.*

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ABSTRACT: Inclusion of fish meal in the diet increases the cost of fish production and this problem can be overcome with replacement of it with local available plant ingredients. The aim of the study was to determine the impact of the complete replacement of fish meal with soybean meal and sunflower meal in the diet of two years old common carp on the growing parameters and proximate composition. Fish were grown in two groups with three replicates in the six earthen ponds each of 1 ha. In the diet 1 was not added feedstuffs of animal origin, while in the diet 2 fish meal was included. The both diets were manufactured using a twin screw extruder. Twelve samples of two years old carp were taken from each group during the harvesting. Also, samples of both extruded diets were taken for analysis. At the end of the rearing period the average harvesting weight was 1622,12 and 1719,2 g; survival rate was 82 and 85%, total stocking density was 300 and 300 kg/ha and harvesting density was 3325,35 and 3612,5,45 kg/ha; feed conversion ratio was 2,3 and 2,1 in common carp fed with diet 1 and diet 2, respectively. The chemical composition was not significantly different between groups ($p>0,01$). These results support the use of adequately processed plant protein sources as important replacement for fish meal in extruded feeds for common carp.
Key words: *formulated feed, soybean meal, sunflower meal, fish meal, common carp*

INTRODUCTION

The main type of fish production in Serbia is the semi-intensive system for cyprinid production, carp being as the major species. In addition to natural food, cereals are supplemented to meet protein requirement. Some fish farms increase meat quality of fish (Ćirković et al., 2011) and production by introducing extruded completed feed mixtures (Ćirković et al., 2005). The cost of inputs per unit of fish weight is higher than in semi-intensive farming, especially because of the high cost of fish feed that contains a high level of protein with a balanced amino acid composition. Fish meal is the first choice as an ingredient in fishfeed production for its high quality of protein with a well-balanced amino acid profile (Gatlin III et al., 2007). The cost of fish meal increased constantly, which caused the need to find alternative protein sources for fish diets. High cost can be overcome by replacing animal origin feedstuffs with local available vegetable-derived protein ingredients. Soybean meal is regarded as an economical and nutritive value feedstuff with high protein content and favourably balanced amino acid profile (Carter and Hauler, 2000) compared to other plant proteins (Gatlin III et al., 2007). In our country, soybean meal is the most commonly used plant protein sources (Ćirković et al., 2005) which could be replaced expensive fish meal in order to develop sustainable and economical carp production. Beside soybean meal, sunflower meal also represents a valuable protein source for fish feed industry. Sunflower meal is an excellent source of linoleic acid, dietary fiber, some amino acids (especially tryptophan), Vitamin E, B Vitamins, and minerals such as copper, manganese, potassium, magnesium, iron, phosphorus, selenium, calcium and zinc. Additionally, it is rich in cholesterol-lowering phytosterols (Ćirković et al., 2002).
The use of plant-derived materials such as legume seeds and different types of oilseed cake, as fish feed ingredients is limited by the presence of a wide variety of antinutritional

substances. Important among these are protease inhibitors, phytates, glucosinolates, saponins tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids, canavanine, antivitamins, andphorbol esters (Francis et al., 2001). Extrusion cooking is used to make plant proten more available to animals through denaturing termolabile antinutritional factors (Barrows et al., 2007), resulting in improved nutrient digestibility and palatability. Heat treatment of soybeans is necessary to denature endogenous trypsin inhibitors and maximize nutritional value, but overheating can damage protein and reduce nutritional value (Barrows et al., 2007).
The aim of the study was to determine the impact of the total replacement of fish meal with soybean and sunflower meal in the diet of common carp on the growing parameters, chemical composition and the amount of total cholesterol of two-year old common carp (Cyprinus carpio L.) in comercial rearing conditions.

MATERIAL AND METHODS

Pond management and diets

Fish were grown in the six earthen ponds each of 1 ha and average depth of 1 m, which were left dry and untreated during winter. Three replicate ponds per dietary treatment were used. Fish were hand-fed twice daily (8:00 and 15:00) for 180 days. In the diet 1 was not added animal feed, while in the diet 2 fish meal was presented in the amount of 32%. The both diet were manufactured using a twin screw extruder. Heat treatment of vegetable origin feedstuffs is necessary to denature termolabile antinutritional factors and maximize nutritional value, but overheating can reduce the availability of protein, reduce protein digestability or cause loss of essential amino acids (Deng et al., 2005) and therefore the extrusion process was strictly controlled.
The fishponds were stocked in April and harvested in October. All fish were reared under variable natural atmospheric conditions. The production of natural food was based on the natural production of benthic and planktonic organisms that were increased by application of agrotechnical measures such as drying of fish ponds during winter, soil treatment, fertilization and adding lime. Livestock manure (2000 kg/ha) was applied to the bottom of empty pond and later biweekly over the water surface (4000 kg/ha during growing season). Agricultural limestone was applied to the bottom of empty pond and over the water surface. The ponds were supplied with well water. The amount of natural food and its influence was not considered because pedologic composition of all 6 ponds was the same, without significant difference, and agriculture measures for improved natural production were the same for the all ponds. Aeration of fish ponds was provided. At the beginning of the feeding trial, a pooled sample of 12 fish was randomly collected to serve as an initial carcass sample.
At the end of the trials, during the harvesting four fish were randomly collected from each pond (n=12, for each dietary treatment) for final carcass analysis. Also, samples of extruded formulated feed were taken for analysis. Fish meal, soybean meal, sunflower meal and other ingredients of the diet were obtained from commercial suppliers.

Growth performance

Growth-performance indicators [specific growth rate (SGR, % weight day^{-1}), feed conversion ratio (FCR), weight gain (WG, %) and survival rate (SR, %)] were measure using following formulas:

SGR = 100 (Ln (mean final body weight)-Ln (mean initial body weight))/time (days);
FCR = dry feed intake (g)/wet weight gain (g);
SR (%) = (Final fish number/initial fish number)*100;
WG = Final body weight (g)- initial body weight(g) (g fish^{-1});

After weighing, twelve samples from each group were stored at a temperature of –18ºC. Before examination, the fish were left at room temperature, in order to partially defrost and

remove skin easily, and separate head, tail, and viscera. Fish fillets were blended (Braun CombiMax 600). To examine total cholesterol content samples were stored in dark plastic bags at temperatures of –18ºC. The meat from dorsal muscles was used for chemical analyzes.

Chemical analysis

Chemical composition of fish muscle tissue and experimental feed mixtures was determined using standard SRPS ISO methods. Protein content was determined by Kjeldahl method (Kjeltec Auto 1030 Analyzer, Tecator, Sweeden). Water content was determined by drying at 103±2ºC to constant weight. For determination of total fat, the samples were hydrolyzed with 4M hydrochloric acid and extracted with petroleum ether by Soxhlet apparatus. Ash content was determined by combustion at 550±25ºC.

Cholesterol determination

Cholesterol in carp fillets (from direct saponification) was measured using HPLC/PDA system (Waters 2695 Separation module/Waters photodiode array detector, USA), on a Phenomenex Luna C18 (2) reverse/phase column, 150 mm x 3.0 mm, 5µm particle size, with C18 analytical guard column, 4.0 x 2.0 mm as described by Maraschiello et al. (1996). The injected volume was 10 µL. The mobile phase was isopropanol-acetonitrile (20:80, v/v) at a flow rate of 1.2 mL/min. Detection was performed at 210 nm and total analysis time lasted 10 min.

Statistical analysis

Intergroup differences were determined ($P<0.01$) using the Student t-test (Statistica 10, StatSoft Inc., Tulsa, USA). The data were presented as means ± SE.

RESULTS AND DISCUSSION

The formulation and chemical composition of experimental diets are given in Table 1. The amount of crude protein and crude fat was almost the same in both mixtures. Diet 1 contained soybean meal in the amount 480 gkg^{-1} and sunflower meal in the amount 100gkg^{-1}, while diet 2 as the main protein source contained fish meal in the amount 320gkg^{-1}.

Table 1. Composition and proximate analysis of the extruded formulated diet

Ingredients	**g kg^{-1} dry diet**	**g kg^{-1} dry diet**
Soybean meal	480	-
Fish meal		320
Sunflower meal	100	
Brewery yeast	50	50
Soybean oil	60	30
Wheat flour	126	20
Corn	140	380
Methionin	1	
Lysine L	3	
Vitamin mix[1]	20	10
Mineral mix[2]	20	10
Chemical analysis	**g kg^{-1} dry diet**	**g kg^{-1} dry diet**
Dry matter (DM)	902.02	895.2
Crude protein (CP)	281.1	281.65
Crude fat (CF)	74.8	73.4
Crude ash (CA)	40.13	73.16
NFE[3]	60.4	57.18
Gross energy (MJ·kg^{-1} DM)[4]	10.66	10.6

[1]*Vitamin mix(mg kg-1 of diet): vitamin B1, 15; vitamin B2, 10; vitamin B6, 20; vitamin B12, 0,15; vitamin K3, 15; inositol, 250; Ca-pantothenic acid, 80; nicotinic acid, 100; folic acid, 1; vitamin H (biotin), 1; vitamin E, 140; vitamin C, 500; vitamin A, 20 000 IU; vitamin D3, 6 000 IU; choline chloride, 1 800, and cellulose was used as a carrier.*

[2]*Mineral mix (mg kg-1 of diet): Cu 20, Fe 40, Mn 30, Se 0.4, Zn 125 , and cellulose was used as a carrier*
[3]*NFE, nitrogen-free extract, g.kg-1 DM = 100 – (CP + CF + CA)*
[4]*Calculated based on the following conversion factors: CP – 24 kJ g-1, CL – 39 kJ g-1, NFE – 17 kJ g-1 13 (Jobling 1994)*

The growth performance of the fishes in terms of weight gain, SGR, FCR and survival rate are presented in table 2. Survival rate of the fishes in the first group was 82% and in the second 85%, which is according recommendation of Ćirković et al (2002). Fish mortality was recorded in all replicates of the treatments and no feed related mortality was observed during the 180 days of the experiment. All growing parameters were better in group which consumed the diet with fish meal, but except in harvesting density, no statistically significant difference was not observed. Among several plant protein sources, soya bean meal was reported to be most efficiency utilized by the fingerlins of rohu when supplemented with methionine and fortified with minerals; though at their higher inclusion levels, the growth was reduced (Khan et al., 2003) Suboptimal amino acid balance, low protein contents and presence of antinutritional factors of plant ingredients seem to be responsible for the decrease in the growth of the fishes at their higher inclusion level in fish diet, especially for marine fish (Tacon, 1997). It has been demonstrated that dietary incorporation of property heated soybean meal improved the growth performance, feed intake, feed efficiency and reproductive performance of common carp (Viola et al., 1983), which is in agreement with our results. Different oilseeds and their by-products usually constitute a major source of dietary protein within aquaculture feeds for warm water omnivorous and herbivorous fish species, so soybean products have been successfully used as partial substitutes for fish meal in diets for Mozambique tilapia (Davies et al., 1989), hybrid tilapia and African catfish (Balogun and Ologhobo, 1989). Sunflower meal has been used as partial substitutes for fish meal in diets for Atlantic salmon (Gill et al., 2006). The majority of published research on the use of plant proteins in fish feeds has focused on the inclusion of soybean meals in feeds for rainbow trout (Gomes et al., 1995, Oliva-Teles et al., 1994; Kaushik et al., 1995) and for salmon species (Hardy, 1996; Carter et al., 1994; Refstie et al., 1998). According to results of these previous studies there are wide range of the amount of vegetable protein sources suggested for the partial replacement of fish meal with vegetable proteins in the diets for salmonids, while in some species, including carp, completed replacement of fish meal with vegetable proteins is possible (Viola et al., 1982; Garg et al., 2002; Khan et al., 2003), which significantly decreases the cost of production.

Table 2. Growth performance of common carp

Variable[2]	**Rearing system**	
	Soybean and sunflower meal	**Fish meal**
Initial number of fish (ind ha^{-1})	2000	2000
IBW (g)	150±16.04	150±24.92
FBW (g)	2027.64±98	2125.08±125
Final number of fish (ind ha^{-1})	1640	1700
Survival rate (%) SR	82	85
Stocking density (kg ha^{-1})	300	300
Harvesting density ($kgha^{-1}$)	3325.33±75.03a	3612.5±100.25b
WG ($gfish^{-1}$)	1877±67.86	1975±89.68
SGR (%·day^{-1})	1.45	1.47
FCR (g·g^{-1})	2.3	2.1

[1]*Data are means ± SE (n = 12). Values within the same row with different letter supercripts differ at p<0.01, IBW, initial body weight; FBW, final body weight; SR, survival rate; SGR, specific growth rate; FCR, feed conversion ratio; WG, weight gain;*

Fillets proximate composition values of the fish at the beginning and end of the 180-day feeding trial are shown in Table 3.

Table 3. Proximate composition of common carp

Variable	initial	Soybean and sunflower meal	Fishmeal
Moisture (g kg^{-1})	829	782.6±6.56	780.5±4.58
Crude protein (g kg^{-1})	141.1a	169.48±2.59	171.42±2.31
Crude lipid (g kg^{-1})	20.9a	37.25±0.35	37.08±4.72
Crude ash (g kg^{-1})	9.8a	10.7±2.01	11.0±1.54
Total cholesterol (mg kg^{-1})	511.3	512.83±11.48	515.75±19.78

[1]*Data are means ± SE (n = 12). Values within the same row with different letter supercripts differ at p<0.01*

No significant differences were found in the chemical composition of carp fillets between dietary treatments, but differences were notably between chemical composition of initial and final fish samples (P<0.01), except for the total cholesterol content of fish fed the both diet and initial sample (P>0,01). Inclusion of plant proteins in the diet of carp has shown that the total replacement of fish meal is feasible.

CONCLUSIONS

These results support the use of adequately processed soybean meal, sunflower meal and other local available feedstuffs as important replacement protein sources for fish meal in extruded feeds for common carp. Fish meal is expensive and mostly imported feedstuff. The use of plant-derived materials as fish feed ingredients are limited by the presence of a wide variety of antinutritional substances, so appropriate heat treatment is necessary.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TP 31011 “Uticaj kvaliteta komponenata u ishrani ciprinida na kvalitet mesa, gubitke i ekonomičnost proizvodnje (The influence of the quality of the components of food for cyprinid fish species on the quality of meat, losses and the profitability of production)”, financed by the Ministry of Science and Technological Development, Republic of Serbia.

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IMPROVING NUTRITIVE VALUE OF BROKEN WHEAT KERNELS BY EXTRUSION

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ABSTRACT: In the technological process of either trade or seed wheat cleaning before drying and storage, organic and inorganic impurities are separated. The amount of separated impurities depends on various factors and can range up to several percent. In the structure of organic impurities, beside broken, shrunken and other cereals kernels, also weed seeds, moldy and smutty grains are present. According to the Serbian Chamber of Commerce, last year 2.093.198 tons of wheat is gathered form 492.989 hectares. In case 2-3% of wheat organic impurities are valorized as feed, this quantity amounts between 42 and 63 thousand tons. It is also important to emphasize that by extrusion process this waste material can be turned into nutritionally and hygiene safe product. Prior extrusion, total count, *Coagulase-positive Staphylococci* and *Clostridium perfringens* were 160.000, <100 <10 cfu/g, respectively. *Salmonella* was not detected in 50 g of the sample, while the total number of yeasts and molds and *Sulphite-reducing clostridia* were 100 and <10 cfu/g, respectively. After extrusion of broken wheat kernels, total count decreased to 17.000 cfu/g, while the number of *Coagulase positive Staphylococci*, *Clostridium pergringens*, *Salmonella* spp, the total number of yeasts and molds, as well as the number of *sulphite-reducing Clostridia* was below the limited value. Extrusion process contributes to physico-chemical changes in the material, primarily in the structural change of starch which is reflected in the increase of sucrose and reducing sugar content in the extrudates. Prior to extrusion, wheat broken kernels were grinded in a hammer mill with screen hole of Ø 4mm, then moisture content of ground material was adjusted to 14.80%. Extrusion was performed on single screw extruder "METAL-MATIK" Beočin, at 90ºC, capacity 150-170 kg/h of grain extrudates. Extrusion head had 8 dies of Ø 10mm. Extruded wheat broken kernels were characterized with moisture, crude ash, crude fiber, crude fat and starch content of 11.81%, 1.68%, 4.43% 1.99% and 69.39%, respectively.
Key words: *wheat broken kernels, organic impurities, extrusion, extrudates*

INTRODUCTION

Food production in the world, and here is one very important problem that arises in both the developed and even more so in underdeveloped parts of the world. Lack of food for the population that each year at the global level significantly increases makes the problem even more important. The solution for increasing the amount of food for humans and animals is the application and use of new technologies and in biotechnology, i.e. bio-industry (Lazarevic et al., 2005). From the technological point of view, here are developed new technological procedures aimed at increasing the nutritive value of food intended for humans or animals, parallel to evaluation of waste products of food and primary agricultural production.
Today in the world in use are different methods for thermal processing of oilseeds and cereal grains such as toasting, extrusion, hydrothermal process, micronization, microwave treatment, and dielectric heat treatment (Thompson, 1987; Sakač et al., 1996; Marsman et al., 1998), but in our country, based on knowledge from practice and literature (Sakač et al., 2001, 2002, 2003) commonly used are extrusion and the hydrothermal process, which provide extruded, or hydro-thermally treated products. Thermal treatments of cereals are used to improve their nutritive, hygiene, physical, chemical and other characteristics, i.e. it increases the nutritive value of some nutritients, improve sensory properties (increases

"sweetness" in the processing of corn) and provides microbiology safe products (Jansen, 1991, Verheul, 1997) and inactivate possibly present heat labile nutrients. Extrusion process leads to changes in the grain complex carbohydrate i.e. decrease in starch content due to its degradation to dextrin. These changes bring about an increase *in vitro* and *in vivo* digestibility of starch, since starch gelatinization provides increased availability of enzymes that break down starch, and parallel to the above, leads to inactivation of the α-amylase inhibitor (Douglas et al., 1990; corp, 1999, Filipovic et al ., 2003). In addition, it was found that extrusion also leads to the increased water absorption and improved hygiene and improvrd physico-chemical characteristics of extrudates (Filipovic et al., 2004, 2005a, 2005b, 2005c). Due to extrusion complex carbohydrates undergo significant physical and chemical changes, which affect the digestibility and utilization of energy from starch. During extrusion, starch particles swell change their solubility in cold water and viscosity of the released amylose and amylopectin in the starch granules. Due to Maillard's reaction, during the extrusion process the content of soluble sugars (reducing) and carbohydrate can be reduced up to 80% (Douglas et al., 1990). Extrusion also leads to significant changes in organoleptic terms, which is directly related to changes in the starch complex.
In this paper the technological parameters for extrusion process, physical-chemical characteristics and microbiology of broken wheat kernels before and after extrusion are presented.

MATERIAL AND METHODS

Extrusion of wheat fracture was performed on the single screw extruder produced by "METAL-MATIK" - Beočin, with 8 dies Ø10mm and a knife at a temperature of 95ºC. Before extrusion wheat broken kernels were grinded in a hammer mill with screen openings Ø4mm and moisture content was adjusted to14.80%.
The basic chemical composition (moisture, crude protein, crude fiber, crude fat and ash) and starch content in the starting raw materials and extrudates was determined by the methods of the Regulations on sampling methods and methods of physical, chemical and microbiological analysis of animal feed (1987). Total sugar content was determined by the Regulations on the physical and chemical methods of analysis for quality control of grain, milling and bakery products, pasta and deep frozen dough (1988).
The total number of microorganisms is determined by the method of BS EN ISO 4833:2008, coagulase positive staphylococci by method EN ISO 6888-1:2008, Clostridium perfringens by BS EN ISO 7937:2008, Salmonella spp by method EN ISO 6579:2008, the total number of yeasts and molds by method ISO 21527-2 and sulphate-reducing clostridia by 15213:2003 ISO method.

RESULTS AND DISCUSSION

Particle size distribution of grinded broken wheat kernels prior to extrusion, is presented in Table 1. Optimum particle size distribution is a prerequisite for the extrusion process. In the process of conditioning added water needs to be quickly and evenly distributed in the conditioner thus enabling optimizing heat treatment in the extruder (Filipovic et al., 2005c). Matz (1993) also stated the importance of particle size of untreated material for the extrusion process in the production of expanded snack products.

Table 1. Particle size of grinded broken wheat kernels

Sieve opening Ø (mm)	Overtails on sive (%)
2,00	0,69
1,25	9,50
1,00	11,90
0,63	31,48
0,25	28,36
0,125	8,84
0,063	6,55
bottom	2,68

The process of extrusion of wheat broken kernels is greatly affecting physical and chemical properties and contributes to reduction of microorganisms in the extrudate. Table 2 shows the chemical composition of wheat broken kernels before and after extrusion at a temperature of 95°C.

Table 2. Chemical compositions of wheat broken kernels prior and after extrusion

Quality parameters	Wheat broken kernels		Extruded wheat broken kernels	
	% in sample	% in d.m	% in sample	% in d.m
Moisture content	14,13	-	11,20	-
Crude protein content	10,49	12,22	10,49	11,81
Fat content	1,72	2,01	1,77	1,99
Crude celullose content	2,29	2,67	3,93	4,43
Ash content	1,38	1,62	1,49	1,68
Starch content	62,77	73,10	61,62	69,39
Total sugar content	3,25	3,78	2,05	2,31

The extruded wheat broken kernels are characterized by significantly reduced moisture content (from 14.82% to 11.20%), making it suitable from the standpoint of keeping quality, i.e. storage. During extrusion, there is a change in the structure of proteins, which often leads to a decrease of protein solubility (Filipovic et al., 2003, 2005) and total crude protein content (decrease in protein extrudates was 3.56% compared to the initial wheat breakage), while the decrease in crude fat content of about 1%, is in accordance with the statement of Venou et al. (2003). Namiki (1990) found increased lipid oxidation in extruded cereals, due to increased contact surface of extrudate with air. In the extrusion process gelatinization of starch occurs parallel with the degradation of its structure and therefore, increases the availability of starch to enzymes in the digestive tract (Douglas et al., 1990, Zhou and Erdam, 1995).

Despite a relatively low temperature of extrusion process (95 0C), it is positively contributing to the reduction of microorganisms in a very short time extrusion (6-10 sec). Very high pressure of extrusion ranging from 30-40 bar, may explain the reduction total number of microorganisms, Table 3.

In the investigated nutrients molds, yeasts and other microorganisms were recorded. For the development of molds feed with high moisture content (over 14%) are particularly suitable. Molds that develop on feed mostly belong to the genera *Aspergillus, Penicillium, Fusarium Altelnaria, Mucor,* and others. (Adamović et al., 2001)

Table 3. Microbiology profile of wheat broken kernels before and after extrusion

Microorganism	Wheat broken kernels	Extruded wheat broken kernels
Total number of microorganisms	160 000 cfu/g	17 000 cfu/g
Coagulase positive staphylococci	< 100 cfu/g	< 100 cfu/g
Clostridium perfringens	< 10 cfu/g	< 10 cfu/g
Salmonella spp	not detected in 50 g	not detected in 50 g
Total number of yeasts and molds	1 000 cfu/g	1 000 cfu/g
Sulphate-reducing clostridia	< 10 cfu/g	< 10 cfu/g

The total number of microorganisms in wheat broken kernels before the extrusion was of 160,000 cfu / g, and after extrusion of microorganisms decreased to 17,000 cfu/g.

CONCLUSIONS

- Process of extrusion of wheat broken kernels at 95ºC leads to physical and chemical changes of the treated material, primarily in changing the structure of a protein and starch, which contributes to a better nutritive value of feed for young animals, fish and pets;
- The process of extrusion of wheat broken kernels contributes to the decreases of the total number of microorganisms thus increases hygiene and feed safety.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III046012 financially supported by the Ministry of Education and Science, Republic of Serbia.

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CHANGES OF CRUDE PROTEIN CONTENT IN LUCERNE PLANT DURING THE FIRST THREE VEGETATION CYCLES

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ABSTRACT: Investigation was performed to observe the changes of crude protein (CP) content in lucerne plant during the first three vegetation cycles within one vegetation season, with the aim to optimize the lucerne utilization in ruminant nutrition. A total of 143 samples were collected from 7 April 2010 (the start of the spring vegetation) to 9 August 2010 (the end of the third vegetation cycle). The sampling was done by cutting a randomly chosen area of 0.1 m^2. Crude protein (CP, expressed in dry matter) was analyzed in all samples with standard Kjeldahl procedure. Samples were collected in all stages of the three vegetation cycles, from the early vegetation to the ripe pod phase.
There were large differences in plant protein content changes during different vegetation cycles. During the first vegetation cycle, which had the highest yield of plant green mass, there was a sudden drop in the protein content that was coinciding with optimal cutting moment. In absolute values, this drop was more than 8 percent points of CP during the 10-day period. Second and third vegetation cycles had slower and more uniform decreases in CP content during vegetation.
Postponing the cutting moment in order to obtain higher yield, during the first vegetation cycle may lead to radical decrease of the nutritive value in lucerne. Adequate and rapid evaluation of the right moment for lucerne cutting is very important in order to obtain quality forage rich in protein.
Key words: *lucerne, crude protein, cutting*

INTRODUCTION

Lucerne (*Medicago sativa L*.) is one of the most important forage species. It has a long life, high yields and is rich in protein. Those characteristics are making it one of the most important feedstuffs on all locations where there are conditions for its production. In the diets for dairy cows it is almost indispensible component. In high yielding dairy cows the lucerne hay, silage and haylage provide important part of crude protein (CP) and calcium. Because of all that, the quality of lucerne hay, silage and haylage is of the utmost importance.
High level of CP in lucerne hay, silage and haylage can be provided only if the plant is mowed at the adequate moment. The classical method for evaluation of precise cutting moment is based on the recognition of certain morphological development phases in lucerne, first of all buttonization and flowering. Depending on the aim, which may be production of the high quality or high yield and quantity, The American Society of Agronomy (Undersander et al. 2004) recommended cutting lucerne in the interval between the beginning of buttonization and the beginning of flowering.
The aim of this work was to determine changes in CP content in lucerne plant during the first three vegetation cycles, and to examine the classical method for evaluation of the right cutting moment, considering its quality in animal nutrition.
There are very few data available in the literature about the changes in CP level in lucerne plant during vegetation, and mostly they show the CP content in the distinctive development (Mejakić et al., 1997; DLG, 1997; UK Ministry of Agriculture, Fisheries and Food, 1992).

MATERIAL AND METHODS

Collection of samples was done on a farm close to Belgrade, from the lucerne field of the Banat cultivar in the third year of production. The field was founded in the spring 2008 and standard agrotechnics were used without irrigation. During 2010 From April 7 to August 9, the total of 143 lucerne samples were collected. Each sample was collected by cutting the

area of 0.1 m^2 at the height of 3.5 cm, which provided between 40 and 200 shoots, depending on the vegetation period. Samples were taken during the first three vegetation cycles at approximately every third day. The interval between two samplings varied depending on weather conditions and vegetation phase. Number of samples in one day depended on the phenophase in lucerne development. In the phases that are most important for animal feeding four samples were collected each day. The beginning of vegetation during the first cut period was the moment when forming of first green shoots was observed (March 21). In the second and third cutting cycles the beginning of vegetation was taken to be the cutting day of the first (May 25) and the second (July 1) swath.
In all three vegetation cycles sampling covered the whole vegetation, from the moment when shoot height was about 10 cm (first sampling) to the phase when lucerne seeds were ripe (last sampling). A total of 72 samples were collected in the first vegetation cycle, 35 during the second and 36 during the third. The first cycle, which happens in the spring, is having the greatest yield of green plant mass and longest lasting vegetation, which is the reason why the largest number of samples was then collected. Collection of samples in the second vegetation cycle was a bit late because of unfavorable weather conditions.
Sampling procedure was following: after the cutting, samples were placed in plastic bags and brought to the laboratory in portable refrigerator, where plants were cut and placed in freezer (-20°C). The estimation of the beginning of buttonization and flowering was done in the laboratory, before samples were chopped, based on the examination of all plants present in the sample. After minimally three days on -20°C the samples were placed in plastic boxes and dried for 48 h in forced draught dryer at 60°C, and the data collected for calculation of dry matter percentage. After the drying, samples were grinded in the laboratory mill with the sieve size of 1 mm. Samples prepared in such way were analyzed for crude protein by Kjeldahl procedure according to ISO standard 5983-2 (2005). Statistical analysis was performed by analysis of variance using Statistica v. 6 software package (2003).

RESULTS AND DISCUSSION

In Serbia all first three vegetation cycles are having substantially different weather conditions. During the first vegetation cycle very low temperatures were recorded and rainy weather, which slowed down lucerne growth. Cold and wet period lasted until the middle of the second vegetation cycle (mid June), while after that moment very high temperatures started, which followed the end of the second and the whole of the third vegetation cycle. The Figure 1 shows the changes in CP% (Dry matter basis) during the three vegetation cycles.
It is possible to observe the decreasing trend in CP% during vegetation. The first vegetation cycle had sudden drop in CP%, until the 40-th day of vegetation, when CP% was stabilized around 17%, and remained at that level until the end of the cycle. The other two vegetation cycles had more balanced decrease in CP%. The period between the beginning of buttonization and the beginning of flowering was shown on the Figure 1 among the dotted vertical lines. In this period during the first vegetation cycle the significant decrease in CP% was observed. On the 34-th day of vegetation, which was the day when buttonization started, plant mass had 26.63% CP while at the beginning of flowering it had 17.12% CP (Table 1).

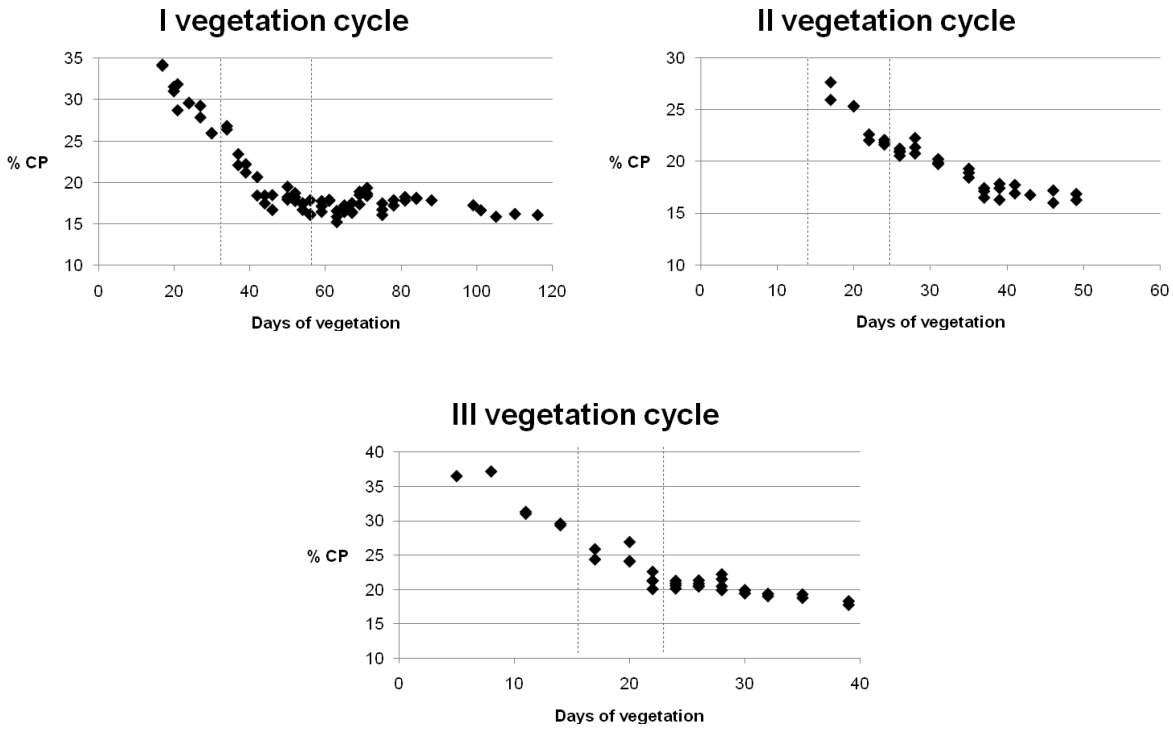

Figure 1. Changes in CP (%DM) during vegetation in I, II and III vegetation cycle

Table 1. Mean value of CP % (%DM) in the beginning of buttonization, flowering and ripe seed during vegetation cycles

Vegetation cycle	Stage of development	Sampling date	Days from beginning of vegetation	Number of semples taken that day	% CP mean value
I	Begining of buttonization	25.4.2010.	34	2	26.63
	Begining of flowering	19.5.2010.	59	3	17.12
	Ripe seed (last sampling)	15.7.2010.	116	1	16.07
II	Begining of buttonization	10.6.2010.	17	3	26.81
	Begining of flowering	17.6.2010.	24	3	21.87
	Ripe seed (last sampling)	18.7.2010.	55	1	16.28
III	Begining of buttonization	18.7.2010.	17	2	25.17
	Begining of flowering	23.7.2010.	22	4	21.35
	Ripe seed (last sampling)	9.8.2010.	39	2	18.08

During the buttonization period of the first vegetation cycle the decrease in CP% was about 9.5% in absolute or 36% in relative value. During the second and third cycle this decrease was much less pronounced – about 5% in absolute value. At the end of the vegetation season when plants had ripe seeds, CP % in lucerne was 16.07; 16.28; 18.08 in first, second and third vegetation cycle respectively (Table 1).

Table 2. Analysis of % CP variation (% DM) during the first vegetation cycle, form midbud to the end of vegetation

Interval	Days	Number of samples	%CP mean value	Sd
Midbud - Beginning of flowering	44-59	19	17.61^{ns}	0.91
Beginning of flovering - End of vegetation	60-116	33	17.30^{ns}	1.00

^{ns}*-Non significant*

Considering only CP% in the first vegetation cycle at May 6 (44-th day from the beginning of vegetation), the CP level was decreased to 17.96%, which in absolute values is more than 8.5%, alredy on the tenth day since the buttonization started. In those samples there was not a one shoot in the flowering phase, while buttonizing plants dominated. Analysis of variance did not show statistically significant differences in samples collected in the following stages: Midbud - Beginning of flowering and Beginning of flowering - End of vegetation (Table 2). Results of this investigation show that during the first vegetation cycle the decrease in CP% reached lowest values during the phases that are recommended as best moments for cutting.

The changes in protein content during the first vegetation cycle were characterized with the sudden decrease in CP during the buttonization phase and beginning of flowering, which is different from the observation of McDonald et al. (1996) according to whom the protein content is comparatively high and declines only slowly with maturity.

According to the recommendations of The American Society of Agronomy (Undersander et al. 2004): when harvesting for the highest yield of high quality, the first cutting should be at bud stage, the second at midbud and subsequent cuttings at 10 to 25% bloom. However, the results obtained in this investigation show that depending solely on the evaluation of the lucerne development phase (from the beginning of the buttonization phase to the beginning of flowering), as a method to determine the moment for lucerne cutting, is of doubtful value if the aim is to produce highest amounts of protein in lucerne hay, silage or haylage. In this investigation this was especially prominent during the first vegetation cycle, when CP% decreased by 8% in the buttonization period alone. Considering that there are inevitable losses when lucerne hay is balled or haylage (silage) is prepared, especially in leaves, this further decreases the CP% of the feedstuff. The shown values are theoretical for lucerne dried in the laboratory, while in the field we can only expect that the losses may be higher.

CONCLUSIONS

The production of lucerne hay, silage or haylage with high protein content requires not only optimization of manipulative procedures, but also the adequate evaluation system for making decision when to cut the green mass. Our investigation showed that relaying only on morphological development phases was not precise enough during the first (and the most important) vegetation cycle. This problem could be resolved by developing a new system for the evaluation of lucerne cutting time, with the aim to obtain the maximum nutritive value from this important forage species.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III046012, financed by the Ministry of Education and Science, Republic of Serbia.

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CONTEMPORARY ASPECTS OF LUCERNE USE IN ANIMAL NUTRITION

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ABSTRACT: An overview of current trends in lucerne use in animal nutrition in different forms: fresh lucerne, preserved lucerne - hay and haylage, or a component of mixtures as dehydrated lucerne and protein-carotenoide concentrate of lucerne juice are presented in paper.
The nutritive value of lucerne is directly affected by the stage of maturity, method of preservation, processing and utilization in animal diets. Based on morphological changes the chemical composition of alfalfa plants can be estimated with high correlations (above 90%). Due to numerous factors that affect on chemical composition of lucerne hay, the nutritive value is highly variable, and using of lucerne hay in rations for ruminants is minimal, according to requirements for optimal digestion and ruminal function. The cut length of lucerne haylage affects average particle length of total mixed rations for ruminants. The physical form and effectiveness of ration is significant parameter for regular rumen function, digestibility of nutrients, and production performances of ruminants, particularly high-yielding lactating cows. The largest reasons for difficulties in preserving lucerne as haylage are in its high buffer capacity, the insufficient content of fermentable sugars and fast degradation of nitrogen compounds. The most favorable results were obtained with simultaneous use of carbohydrate supplements with homofermentative lactic bacteria for wilted plant material.
In spite of relatively high content of crude fiber, lucerne may be significant for nonruminant nutrition after specific treatment and processing, particularly for hen feeding with the purpose of yolk color modification. Lucerne juice may be particularly important for this purpose because it is a protein-carotenoide concentrate.
It can be concluded that lucerne still remains one of the main feedstuffs due to its high nutrition value and exceptional biological characteristics.
Key words: *lucerne, preserving, processing, ruminants, nonruminants, nutrition*

INTRODUCTION

Lucerne is the second most important animal feedstuff in Serbia, after corn. According to the statistical data Statistical Office of the Republic of Serbia, in the May 2010 the total of 3 065 745 ha was seeded in Serbia, of which 187 286 ha (6.11%) was seeded with lucerne. This shows the importance of this culture, considering that it is perennial plant. Lucerne has high nutritive value (high protein content, calcium and carotene), high yields (to 40 tha^{-1}; Đorđević and Dinić, 2007), longevity (5-7 years of production), several cuts during a vegetation (to 8 when watered) and various opportunities for its use (as fresh forage, hay, silage of haylage, meal and protein concentrate). However, lucerne yields were just 5.837 tha^{-1} (Statistical Office of the Republic of Serbia) during the 2011 in Serbia, which is significantly below its potentials. The main reason for that is in inappropriate agrotechincs. It is also evident that lucerne is usually cut late, when its nutritive value is reduced. Lucerne contains some antinutritive substances (saponines, estrogens and other) and when fresh it easily causes bloat in ruminants. Due to its high fibre content its use is limited in nonruminants. The important imperfection in lucerne is that it cannot be produced on acidic soils (Kammes et al., 2008). In spite of all that, the majority of lucerne is used as hay in Serbia, which is the most variable feedstuff according to its nutritive value (Đorđević et al., 2010, 2011).

LUCERNE AS FRESH FORAGE

All methods of lucerne conserving are producing certain losses in nutritive value, due to mechanical loss of chemical transformations of nutrients. The use of fresh lucerne as pasture

or cut and given to animals in the stall is good solution for those problems. However, lucerne is rarely used as pasture because it does not stand well animal trampling. Some low breeds were selected for that purpose in the past (Mejakić et al., 1997). The other problem with the use of fresh lucerne is that it is somewhat limiting dry matter and energy intake (Bargo et al., 2002), and because of that the cow ration needs to be supplemented with concentrates and conserved feeds. The bloat is also a serious threat with lucerne, and also low pH value of rumen fluid, low content of NDF and high ammonia concentration in the rumen. The use of Monensin in dairy cows fed on lucerne pasture diets is used to reduce methanogenesis, and it results in the increase of milk yield with decrease in milk fat content (Gallardo et al., 2005). Modern concepts in dairy cows feeding are based on conserved forages and concentrates, combined in „total mixed rations" (TMR), where fresh forage is not normally used (Đorđević et al., 2006).

LUCERNE HAY

The most variable type of hay is produced from lucerne, considering its nutritive value and chemical composition. Therefore it may limit the milk production in high yielding cows, if used as sole or dominant forage. It is also expensive feedstuff, but minimal amounts of hay with its physical properties are indispensable for normal functioning of the ruminant digestive system (Grubić et al., 1999). The combination of lucerne hay and corn silage in dairy cow diets is optimizing their dry matter (DM) and energy (NEL) intake, which leads to the increase in production of milk, protein and lactose (Kowsar et al., 2008). High amounts of lucerne hay or haylage in the diets of prepartum cows may lead to milk fever due to high content of calcium cation (Grubić and Adamović, 2003). This type of feeding is common on small family farms in Serbia. According to Stojanović and Grubić (2008) the Dietary Cation-Anion Difference (DCAD) value for lucerne hay is +431 mEq/kg DM, which significantly contributes to the DCAD value of the whole diet. The diets for dry cows should have negative DCAD value. It is therefore recommended to add a mixture anionic salts (Đorđević and Dinić, 2011) if lucerne is the only available hay. Goff et al. (2007) used chlorine fertilizers (NH_4Cl and $CaCl_2$, and their mixtures) in the amounts of 56, 112 and 116 kg Cl/ha, and discovered the increase in chloride content in lucerne forage from 0.52% to 0.77, 0.87 and 0,89%. These results show that it is possible to add chlorine to the soil and increase its content in the obtained forage, which is decreasing DCAD value in lucerne hay or haylage.

LUCERNE SILAGE (HAYLAGE)

High moisture content in the moment of cutting and high buffer value are the main problems for successful ensiling of legumes, and this was the reason for several experiments performed during the past couple of decades (Đorđević and Dinić, 2003). Today the more attention is also given to the changes nitrogenous matters which occur during the ensiling process (Đorđević et al., 2004). In living plants 75 – 90% of total nitrogen is in the form of true protein, while in silages it is just 30 – 50%, according to Slottner and Bertilsson (2006). Compared to other plants from the *Fabaceae* family lucerne has more soluble nitrogen matters. Protein solubility is in positive correlation with ruminal degradability (Wattiaux, 1994), which may reduce their utilization or lead to health problems in animals. According to Broderick (1995) other legumes have less soluble proteins compared to lucerne, due to higher presence of condensed tannins. Such results were experimentally obtained by Albrecht and Muck (1991) for sainfoin, lespedeza, and *Lotus pedunculatus*. According to the same authors, species such as red clover or *Kura* clover do not contain condensed tannins, but their proteins have low solubility. The lesser degree of proteolysis in red clover silages was explained with the presence of soluble enzyme polyphenol-oxidase, which in presence of oxygen reacts with O-diphenol creating very reactive O-quinone, which creates polymers with other molecules such as proteins (Getachew et al., 2009; Graber, 2009; Lee et al., 2009).

Various methods are used in order to maximally control the process of nitrogen matter degradation during the legume ensiling process, such as wilting, carbohydrate stimulation, inoculation and chemical conserving (Nadeau et al., 2000; Guo et al., 2008). Aside from these technologies the cultivars of legumes are selected to have lower degradability (Broderick et al., 2004), and also genetically manipulations were done with the same purpose (Getachew et al., 2009).

On larger farms in Serbia lucerne hay and haylage are given in a mixture with other feedstuffs as TMR. One of important factors for the use of forages is their cutting length which is important for their physical effectiveness. The minimum content of physically effective fibre (PEF) is needed for chewing activity, saliva production and contractions of the reticulo-rumen (Stojanović et al., 2010). The content of PEF in the complete dairy cow ration has influence on animal health, ruminal fermentation, intake and digestibility of the diet, and also on yield and chemical composition of milk (Bhandari et al., 2008). Stojanovic et al., 2012) in their experiment on dairy cows discovered that when forage feed particles are reduced, the feed conversion ratio is improved, and also digestibility and milk yield, while protein content in the milk was reduced (Table 1).

Table 1. Effects of reducing forage cut length on yield and composition of milk and feed conversion of cows in early lactation (Stojanovic et al., 2012)

Item	Treatment				SEM[1]	p-values
	Long	Medium-long	Medium-short	Short		
Actual milk, kg day^{-1}	35.62a	35.64a	36.23ab	38.36b	0.39	0.027
4% fat corrected milk, kg day^{-1}	32.82ab	31.39a	31.22a	34.71b	0.40	0.005
Milk fat, %	3.50b	3.21ab	3.10a	3.35ab	0.036	0.001
Milk fat, kg day^{-1}	1.25b	1.14a	1.12a	1.28b	0.013	≤ 0.001
Milk protein, %	3.11b	3.03ab	3.00ab	2.99a	0.013	0.004
Milk protein, kg day^{-1}	1.11a	1.08a	1.09a	1.15b	0.01	≤ 0.001
Milk fat: protein ratio	1.13b	1.06a	1.03a	1.12b	0.013	0.037
Dry matter intake, kg kg^{-}1 milk	0.64b	0.64b	0.62ab	0.60a	0.004	0.001
Dry matter intake, kg kg^{-1} 4% fat corrected milk	0.70ab	0.73b	0.73b	0.68a	0.006	0.001

[1]*SEM: Standard Error of the Mean. a, b, c Means in the same row with different superscripts differ (p<0.05)*

LUCERNE MEAL

Dehydration and processing of lucerne with high temperatures in the process of meal production has little effects on content and composition of proteins, their biological value and biological value of their amino acids. Along with high protein content lucerne meal has high level of minerals, carotene, B complex vitamins and also C, K and E vitamin. By sieving lucerne meal the fraction for nonruminant feeding is obtained, with 23-25% protein and 15-17% crude fibre. Delić et al. (1976) were not able to substitute soybean meal even with double amounts of de-cellulosed lucerne meal (27% protein and 14% fibre), in the feeding of fattening pigs. They explained those results with low digestibility of lucerne. Arshad Al-haweizy and Yasin Al-Sadrady (2007) included lucerne meal as 4, 8, 12 and 16% of the mixture for laying hens and discovered that with the hens' age and level of the meal the conversion ratio is decreased. Because of negative effects on diet digestibility the use of lucerne meal nonruminant feeding is decreasing. Also, the correction of amino acid profile in the diets is today applied easily with synthetic amino acids, which is decreasing the importance of lucerne meal (Đorđević et al., 2007).

Lucerne meal has higher content of undegradable protein compared with fresh lucerne or hay (Grubić et al., 1994). This may be the reason for its use in dairy cow nutrition, in order to obtain the required balance between degradable and udegradable proteins in the diet.

LUCERNE PROTEIN CONCENTRATE

Protein concentrate produced from the fresh lucerne juice is important protein source, but also of carotene, which is givig color to some animal products (broiler chick skin, egg yolk). In near future it may became important protein source for human nutrition, about which there were some experiences from the Second World War (Đorđević et al., 1997).
The procedure of juice production is consisting of sqeezing the fresh chopped green mass with special presses, while proteins from the juice is extracted by coagulation and filtration. Domestic product is alowing production of 9.09% protein concentrate from lucerne dry matter (Sredanović et al., 1991). Lucerne protein concentrate has high amount of essential amino acids, xantophyle, β-carotene, α-tokoferol, and some stimulative and other biogenic materia. Kuzmicky and Kohler (1977) discovered that when protein concentrate from lucerne leaves (62.8% protein) was included 2.5, 5.0, 7.5 and 10.0% in the diets of broiler chicks there was no significant differences in their body mass and feed conversio ratio. Olvera-Novoa et al. (1990) replaced 35% of fish meal in mixture for tilapia (*Oreochromis mossambicus*) feeding with protein concentrate from lucerne leaves and there was no depression in their growth.

CONCLUSION

High nutritive value of lucerne is most eficiently utilized in the form of conserved feedstuffs. Along with hay, silages and haylages have increasing importance for high and economic production in cattle, in combination with feeds that are cheap energy sources. In the world there is a trend of reduction of hay production and use, and increase in the production of lucerne silage/haylage. Lucerne hay and haylage are indispensable components of total mixed ratios for dairy cattle, with particular care taken to the chopping length (physical effectiveness). Problems with production of quality lucerne silage and haylage can be solved with the use of various methods of fermentation induction or stimulation, also with the reduction of proteolysis. Lucerne meal has limited use as protein-vitamin additive in nonruminant diets, because of its high fibre content. In the near future protein concentrate produced from lucerne juice may be important source of nutrients not only for animals but also for humans as well.

ACNOWLEGDEMENTS

The autors are grateful to the Ministry of Education and Science of the Republic of Serbia which financed this Project investigation within the III-46012.

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IMPORTANCE OF COPPER AND SELENIUM IN NUTRITION ON HEALTH AND PRODUCTION TRAITS OF GOATS

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ABSTRACT: Mineral deficiencies in goats in many countries are caused by low or variable contents due to season and maturity of plants and low digestibilities. The reliability of body tissues as an indicator of deficiencies varies greatly with mineral elements. Major performance improvements have been achieved, nevertheless, where mineral supplementations were applied correctly. Requirements of macro- and microelements, or minerals for short, are based on evidence of metabolic functions, which are structural and/or catalytic.
Mineral supplementation on this basis has yielded improvement in milk production, reproduction, feed intake and reduced heat stress in other species. It also has been pointed out, that even in the best studied species, cattle, there is no academic agreement as to the feeding recommendation levels of minerals, and there is less agreement and knowledge about the other less studied species, such as the goat. Copper is an essential element for the synthesis of hemoglobin, the iron absorption in the small intestine and the mobilization of Fe in body depots and oxidation. The function of selenium in metabolic processes is a close related to vitamin E. So, diseases caused by deficiency of Se can atribute to deficiency of Se and vitamin E. Deficiency in Se caused a great numbers of diseases with goats: degeneration of heart and sceletal muscles, more affinities to dysentery, disorderes in reproduction etc
Key words: *the goat, minerals, production traits*

INTRODUCTION

Providing animals with trace elements is done through food, special supplementation (premix) or in water. The intensive production of adding them is mandatory, because only thus can provide in sufficient quantities for optimal health and performance (Memiši et al., 2004ab; Memiši and Bauman, 2007). Minerals activate enzymes, are essential cofactors of metabolic reactions, act as carriers of proteins, regulate digestion, respiration, water balance, muscle reactions, transmission of nerve, influence and maintain skeletal strength, balance pH, and even mental balance, protect against disease, antagonists were or synergistic and other elements play a vital role in the resistance, adaptation and evolution of new breeds and lines (Solaiman et al., 2001).
Regardless of the fact that some of the trace elements present in sufficient quantity in food, often occurring subclinical or clinical signs of deficiency, because their efficiency varies and is a trace element found in unusable form. Resorption of trace elements depends not only on content in food, but also of animal age, electrochemical reactions in the intestine and form in which the trace is located. Depending on the type of soil, none of them provides an optimal level of minerals. In addition, plant species, their exclusion maturity and climatic conditions have also an important role in the content of minerals in kabastoj food. When talking about the lack of minerals in the diet of goats, then the first thought that their depletion leads to severe clinical manifestations and the emergence of easy identification of symptoms (Memiši et al., 2003,Memiši and Bauman, 2003d).
However, as noted in Figure 1, subclinical manifestations of the lack of certain minerals that are reflected in reduced immunity, growth, fertility, will be manifested long before the classic clinical features (Farzana, 2005). Subclinical deficiency of minerals in the diet is common and occurs in flocks of goats, however, producers are very difficult to notice their absence.

Animals seem to have normal growth, development and reproduction but it is at a lower level of a normal production. For these reasons, the diet of goats in all stages of the production cycle is commonly used supplements of minerals in order to maintain adequate and efficient production. For this purpose, commonly used salt minerals and oxides, carbonates, chlorides and sulphates. Today it is increasingly beside the form of inorganic minerals, using the so-called "chelate" form, ie, organically bound trace elements (Memiši et al., 2004a, 2007; Memiši and Bauman, 2003a,b,c).

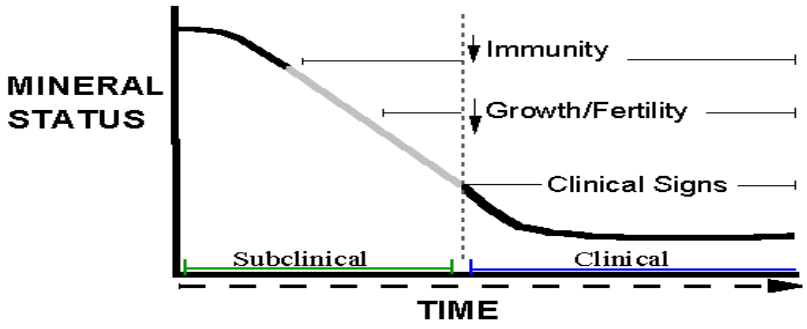

Figure 1. The relationship between mineral status and onset of subclinical and clinical disease symptoms

Requirements of goats in copper and selenium

Quantity of some minerals in foods is not as important as there's availability for the animal itself. This availability varies widely, and it affects a number of factors such as the form in which there is an element in the food, the stage of development of plants, the presence of other minerals and substances in foods that bind them and make available, then age and sex of animals, etc. In order to prevent deficiencies of certain mineral elements in nutrition of goats, there are many possibilities and application of certain procedures and methods to eliminate them and these are primarily: the treatment of forages with different fungicide, adding elements in the processing of land used for production of forage, use ready-made and complete mineral mixture in diet, and ultimately increase the organic reserves in the body of animals - goats can be made, giving injections or capsules with a slow and prolonged action.

Significant differences in mineral requirements between species, among other items, established for Cu and Se (Devendra, 1990). The lower the minimum needs of goats in selenium, depending on the stages of production (Kessler, 1991; Haenlein, 1991) ranges of 0.1 - 0.2 mg / kg dry matter intake. Chronic poisoning with Se in the skin (as in sheep) occurs when during the long period of time, consume feed containing more than 3 ppm Se in dry matter. Excess Fe, Cu and Ca in the diet increase the need to selenium.

Table 1. Effect of Cu (mg) supplementation on growth performance of goat kids (Solaiman et al., 2004)

Days	**0 Cu**	**100 mg Cu**	**200 mg Cu**	P- value
	Average daily gain, g			
28	156.6	144.5	167.7	0.61
42	147.6	156.6	134.1	0.39
56	137.4	149.6	129.9	0.19
70	134.9	153.1	119.2	0.02
84	131.7	147.6	116.6	0.01
98	129.2	147.3	122.5	0.04

When it comes to copper, the symptoms of toxicity in sheep can be observed with 10-20 mg Cu/kg DM entered the food, while cows submitted to 100 mg Cu/kg DM. The diet with the addition of higher levels of 100 mg Cu as a positive influence on the increase daily gain in

the young category and contributes to the improvement of immune function in goats (Table 1).

Research around the world have shown that in some countries as regards the presence of certain minerals in the soil there are different variations in terms of their deficits and surpluses, which could help in directing and creating programs to resolve problems (Solaiman et al., 2004). Such examinations of soil and plants should be placed in connection with the specific characteristics of metabolism of various species, and accept that the analysis of animal tissue is definitely the right diagnostic measures, but different tissues have different affinities to the macro-and micronutrients, and some of them do not have, and so their value as indicators is different, as is the case with these two mineral elements (Table 2). Thus, the status or supply with copper and selenium in the body skin is best tested through the coat (and over its content in blood plasma), which is one of the best indicators of the presence of this mineral in the body skin. In addition to hair good indicators supply the trace elements can be either internal organs (Anke et al., 1988), which are primarily the liver, then the brain, spleen, muscle, milk and others.

Table 2. Significant differences Cu i Se in tissue contents in goats, mcg/kg DM (Anke et al., 1988)

Minerals	Tissue	(n=31)/ Controls	(n=22)/ Cu and Se deficient
Cu	Brain	14.0	6.0
	Hair	4.7	3.0
	Plasma	0.7	0.4
	Liver	10.0	5.3
Se	Muscle	387	146
	Plasma	130	36
	Hair	350	150
	Milk	247	93

Copper is required goats at a level that is usually included in the diets of dairy cows, in contrast to sheep, which are sensitive to such levels, so that in such cases for them to develop and toxicity (Solaiman et al., 2006). Copper is an essential element for the synthesis of hemoglobin, the iron absorption in the small intestine and the mobilization of Fe in body depots and oxidation. Sheets and certain browsing contain higher levels of copper than stem forage, but their concentration decreases with the progress of maturity of plants for 40 to 60%. Deficiencies of copper in goats can be prevented by adding 0.5% copper sulphate in a mixture of minerals.

Lack of copper causes sudden weight loss goats, depigmentacion and hair loss, anemia, retarded growth, reduced milk production, diarrhea, the occurrence of hidden oestrus, the bones become brittle and deformed and others. If they enter the higher concentration of Cu can accumulate in the liver without major problems in animal health. Deficit of Cu in goats (less than 2 mg Cu / kg DM / day to 8 mg of Cu in the control group of goats, increased the content of Zn in the liver and ovaries and reduced food intake by 50%. In the burden of the Cu, goats that were fed higher levels of copper, had a 6 to 9 times lower levels of copper in the liver in the control group in the study, indicating individual differences between animals in the exploitation and resistance to the toxicity of Cu (higher levels in ration), which might be related to hepatic soluble protein that binds Zn for Cu (Zervas et al., 1989). However, the diet with high levels of sulfur (> 0.35% based on dry matter), molybdenum (> 2 ppm of molybdenum in food or the ratio of Cu: Mo <5:1), Fe (more than 250 ppm), Ca, Zn, Mn and Co can significantly reduce the absorption of copper (Solaiman et al., 2004).

For selenium deficiency in the diet of goats can be suspected in areas of his deficits in the soil, when the goats appeared various reproductive problems, early embryo mortality, recurrent estrus, retention of the placenta after giving birth to kids, metritis, weak newborn kids, degeneration of cardiac and skeletal muscle (white muscle disease), diarrhea, dyspnea (difficulty breathing), etc.

Intramuscular injection in goats with preparation selenium - vitamin E, a month before kidding can prevent the occurrence of these symptoms in the newborn kids, but adding 0.2 ppm of

selenium in the diet of goats provides stable protection during the entire production cycle. Status of selenium in the body skin is the best way to test his analysis in milk or blood, and in hair samples. Selenium in minimum amounts in the diet of goats needed to avoid problems in the reproduction of goats and retention of the placenta, then important for the functioning of the immune system, whereas in its operations is closely related to vitamin E.

Table 3. Requirements of goats in Se (NRC, 2007)

Physiological state	Requirements
Maintenance	0.015 mg/kg DMI + 0.083 mg/AC
Growth	0.5 mg/kg LWG/AC
Pregnancy	0.0021 mg/kg LBW/AC
Lactation	0.10 mg/kg MY/AC
Mohair	0.38 mg/kg

DMI- dry matter intake; AC-Apsorption coefficient (forages-0.31 and concentrates 0.60); LWG – Live weight gain kg/d; LBW – Litter birth weight, kg; MY – Milk yield, kg/day

Se be absorbed from the diet to a much greater extent than eg. Fe, Cu, Zn and Mn, and does not depend on the chemical form of selenate, selenite or selenides (Hart, 2008). Goats are related to the casein in milk and about 3% of ingested Se occurs in the milk of the correlation coefficient of r = 0.7. As for the metabolism in the body, it can absorb and release the body to the fullest extent through the lungs (10 to 50%), but most of it is expelled through the kidneys, while the fecal excretion of about 10%.

Because goat is largely used for feeding browsing of shrubs, bushes and trees, weeds and grasses that do not belong, and which are largely studied analytically, it is necessary to know its chemical composition (Devendra, 1990). When it comes to the use type of travel and poor grass in the diet of goats, one should bear in mind that many species of plants for the browsing of limited value because of one or more inhibitors that can bind, or otherwise to prevent the use of nutrients (mainly minerals) from them. Based on this fact is the addition of minerals in the diet of domestic animals (including goats) which largely led to the improvement of milk production, reproduction, food intake and reducing stress due to heat in other species.

On the basis of specific experiments on goats, the wording needs in the minerals is less dependent experiments were conducted on sheep and cattle (Haenlein, 1991; Kessler, 1991) (Table 4).

Table 4. The latest minimum mineral requirements of goats (Kessler,1991)

Minerals	
Cu	8 - 10 - 23 mg/kg - DM/day
Se	100 - 200 mcg/kg - DM/day

CONCLUSION

High production and milking goats requires a larger number of macro - and trace elements due to which growth and the need for them. Their adequate balance in the diet of goats is difficult because of specificity in the diet of goats, respectively, for the use of nutrients whose nutritional value not known. Goats are tolerant of high doses of Cu and Se with no obvious bad effects on their health, in contrast to sheep and cattle. Lack of copper causes sudden weight loss goats, hair loss, anemia, retarded growth, reduced milk production, diarrhea, the occurrence of hidden oestrus, the bones become brittle and deformed. Therefore, adequate nutrition of goats requires the maximum balance of nutrient meal, which is achieved by using different feed and using mineral mixtures. Diet with higher levels of Cu addition of 100 mg per day has effect on daily weight gain in younger categories and contributes to improved immune function of goats. The lowest minimum of goats need in selenium, depending on the stages of production, ranges from 0,1 to 0,2 mg/kg of ration dry matter. Therefore, adequate

nutrition of goats requires maximum balance of nutrients, which is achieved by usage of different feed and the usage of mineral premix.

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EFFECT OF RAPESEED MEAL ON NUTRIENT DIGESTIBILITY IN BROILER CHICKENS

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ABSTRACT: Rapeseed meal (RSM) is a good protein source for animal nutrition, but there are some concerns about its effect on digestibility and gut health, especially in young animals such as broiler chickens. Study was carried out to determine the effect of rapeseed meal in broiler chicken diets on nutrient digestibility. Three groups of chickens were formed and fed either with corn-soy based diet (control group) or with inclusion of 10% (RSM 10%) or 15% of rapeseed meal (RSM 15%) respectively. Feed was composed to meet nutritional requirements of used broiler strain and balanced to same energy and crude protein level. To determine digestibility, 20 Ross 308 male chickens of average weight were used per treatment. Acid insoluble ash was used as marker. At 21 days of age, chickens were sacrificed to obtain samples for determination of ileal digestibility. Digestibility of dry matter, crude protein and energy was determined. No significant differences (P>0.05) were observed in any measured digestibility parameter. It is concluded that addition of up to 15% of rapeseed meal in well balanced diets of young broiler chicken does not have an adverse effect on digestibility of nutrients.
Key words: *rapeseed meal, digestibility, broilers*

INTRODUCTION

World production of rapeseed *(Brassica napus)* is growing (Marinković et al., 2010) driven by increased use of rapeseed for production of biodiesel and oil for human consumption. Rapeseed is also one of the major protein sources in European organic livestock production, since climate in large part of Europe is not suitable for soybean growing (Blair, 2011). It can be assumed that rapeseed production in Serbia will increase in future (Popović, 2010), and that larger quantities of rapeseed meal for animal feeding will be available.

Rapeseed meal is by-product from the manufacture of rapeseed oil, obtained by solvent extraction of rapeseeds, and is a good protein source for animal nutrition. It usually contains 34% of crude protein (INRA-AFZ, 2002). Amino acid profile of rapeseed protein is good. It contains 1.8% of lysine (more than sunflower meal, and less than soybean meal) and 1.51% methionine and cystine (more than both sunflower and soybean meal). It has more fibers then soybean meal, but less then sunflower meal (12.4% of crude fiber, 28.3% of neutral detergent fiber and 19.6% of acid detergent fiber). Energy content of rapeseed meal for different domestic animals, including poultry, is also between values for sunflower and soybean meal, two protein sources commonly used in Serbia.

In the past, main problems with use of rapeseed and its products in human and animal nutrition were erucic acid and glucosinolates, but contents of these compounds are reduced to very low and safe levels in modern group of cultivars called canola or double zero rapeseed (Leeson and Summers, 2001). Rapeseed meal also contains some other compounds that can possibly affect its nutritional value and its use in animal nutrition, like tannins, phytic acid and, in particular, sinapic acid and sinapine. Sinapic acid and sinapine are compounds that are responsible for unpleasant odor of eggs from brown-shelled egg layers fed rapeseed meal (Leeson and Summers, 2001). Sinapine generates bitter, astringent and pungent taste and irritating flavor (Trozyńska, 2004), but it seems that it does not negatively affect feed intake in broiler chickens (Qiao et al., 2008) or laying hens (Johnson et al., 2008). Johanson et al. (2008) even found positive effects of sinapine on

energy and protein digestibility in laying hens. On the contrary, Qiao et al. (2008) found that sinapic acid can reduce digestibility of amino acids when fed in larger quantities, but concluded that sinapic acid and sinapine are not toxic to broilers. Rapeseed also has high level of sulfur that can disturb mineral balance and induce leg problems in broilers (Leeson and Summers, 2001). However, main factor limiting the use of rapeseed in animal nutrition is its high level of fibre, resulting in low energy value and suboptimal use of proteins (Slominski et al., 1999).
Aim of this study was to determine if broiler diets with 10 and 15% of rapeseed meal can be used without negative effects on digestibility of nutrients.

MATERIAL AND METHODS

Six hundred day-old Ross 308 chickens were obtained from local hatchery. Chickens were randomly distributed into 15 pens, 40 chickens per pen. Pens were randomly assigned to 3 treatments, with 5 replicates per treatment. Stocking density was 16 birds per m^2 and wheat straw was used as litter. Feed and water were supplied *ad libitum.* Air temperature was adjusted in accordance with the recommendation of the strain producer (Aviagen, 2009). The three treatments were as follows: Control - diet without rapeseed meal; RSM 10% - diet with 10% of rapeseed meal; RSM 15% - diet with 15% of rapeseed meal. Rapeseed meal used in diets was made from “canola quality” rapeseed. Composition of diets is shown in table 1. All diets were balanced to same level of crude protein and ME.
At 14 days of age chickens were weighted, and 20 males of average body weight were taken from every treatment, four from every pen. They were randomly (within treatment) distributed into wire mesh cages, five chickens per cage. Four cages per treatment were used, 12 cages for the whole trial. Water and feed were again supplied *ad libitum*. After two days of adaptation period, marker was added in diets. Celite was used as a source of marker - Acid insoluble ash (AIA). One percent of Celite was mixed with 99% of diets. Diets were sampled after Celite addition, and analysed. After six days of consuming diets with Celite, broilers from cages were slaughtered and small intestines were removed. Digesta samples for digestibility analysis were obtained from the most distal 10 cm of ileum. This part represented approximately one fourth of total ileum length. The last centimetre of ileum before ileocecal junction was not used to avoid mixing ileal and cecal digesta. Digesta samples were obtained by gentle squeezing to avoid contamination of samples. Samples from all birds from one cage were mixed together. Immediately after collection, samples of digesta were frozen, and they were kept frozen until analysis. In diets and ileal digesta samples, dry matter, AIA, crude protein and gross energy content were analysed. AIA was analysed according to *Vogtman et al*. (1975). Crude protein was analyzed using Kjeldahl procedure. Gross energy content was analysed using bomb calorimetry (*c200 Calorimeter, IKA®*). Apparent ileal digestibility for dry matter, crude protein and energy, was calculated with the following formula:

$$Digestibility = 1 - (AIA_{diet} \times N_{digesta}) / (AIA_{digesta} \times N_{diet})$$

where AIA_{diet} is percentage of AIA in diet, $N_{digesta}$ is nutrient parameter (percentage of dry matter, percentage of crude protein or gross energy expressed as MJ/kg) in ileal digesta, $AIA_{digesta}$ is percentage of AIA in ileal digesta, and N_{diet} is nutrient parameter in diet. All values are expressed on dry matter basis.

Table 1. Composition of experimental diets

	Control	RSM 10%	RSM 15%
Diet composition (g/kg)			
Corn	490	450	430
Full fat soybean	138	141	150
Soybean meal	280	210	168
Rapeseed meal	0	100	150
Yeast	30	30	30
Soybean oil	18	27	30
Monocalcium phosphate	15.5	13.5	12.5
Limestone	14	14	15
Salt	3	3	3
DL-methionine	1.5	1.5	1.5
Vitamin and mineral premix	10	10	10
Calculated nutrient composition			
AME (MJ/kg)	12.8	12.8	12.8
Crude protein (g/kg)	226	225	226
Crude fibre (g/kg)	41.5	46.7	49.2
Lysine (g/kg)	12.6	13.0	13.2
Methionine (g/kg)	5.1	5.3	5.4
Ca(g/kg)	9.0	9.2	9.7
P, total (g/kg)	7.6	7.8	8.0

All procedures were performed following standard ethical norms and birds were not subjected to undue stress. Digestibility data were analyzed by one way ANOVA, with using general linear models. Results were considered significant when $P<0.05$. Analysis was performed using Statistica software (StatSoft Inc., version 8.0, 2008).

RESULTS AND DISCUSSION

Observed ileal digestibility values are shown in Table 2. Digestibility of dry matter, crude protein and energy were numerically lower in groups consuming rapeseed meal, but these differences were far from statistical significance. Digestibility of all three parameters were lower in the RSM 10% group then in RSM 15% group.

Table 2. Ileal digestibility of dry matter, crude protein and energy of diets with or without rapeseed meal (%)

	Control	RSM 10%	RSM 15%	SEM	P value
Dry matter	68.6	66.4	67.6	0.92	0.662
Crude protein	80.0	77.6	79.0	0.78	0.516
Energy	73.4	71.1	71.9	0.87	0.582

SEM-standard error of mean

Dry matter digestibility values were similar to values determined by Juanpere et al. (2005) using diets based on corn and soybean, chickens of similar age and total collection method. Protein ileal digestibility values for all treatments were within usual range for this method, somewhat lower then values reported by Marsman et al (1997), higher then values reported by Schnietz et al. (1998) and Hernandez et al. (2004), and very similar to values reported by Namkung and Leeson (1999). Lee at al. (1995) demonstrated that rapeseed meal have higher amino acids digestibility (and similar metabolizable energy level) compared to flax meal, when fed to White Leghorn roosters. Digestibility of some amino acids and level of metabolizable energy is higher in expeller extracted rapeseed meal than in solvent extracted canola meal, when fed to three weeks old broilers (Woyengo et al., 2010). Apparent metabolizable energy was lower in diet with 30% of rapeseed meal then in diet with 20%, but apparent digestibility coefficient of nitrogen was not significantly affected with level of rapeseed meal in diets of 43 days old broiler chickens (Mushtaq et al., 2007). Also, enzyme

(xylanase and glucanase) supplementation did not have effect on examined parameters (Mushtaq et al., 2007).
Observed digestibility levels indicate good intestinal health and undisturbed digestion in broilers consuming rapeseed meal. Lack of negative effects of rapeseed meal on nutrients digestibility of broiler chickens in this trial is a result of: (1) using meal from "canola quality" rapeseed with low level of antinutritive factors; (2) using moderate levels of rapeseed meal (10 and 15%) compared to much higher levels in some other studies (Mushtaq, et al., 2007); and (3) using well balanced rations, where rapeseed meal was combined with increased levels of energy dense feeds-soybean oil and full fat soybean. These results are in agreement with performance study reported by Ahmad et al. (2007), and their conclusion that high quality canola meal can be included in broiler diet up to 20% without negative effects.

CONCLUSIONS

From the results of this study, it can be concluded that addition of up to 15% of low erucic acid, low glucosinolate, "canola quality" rapeseed meal in well balanced diets for young broiler chicken does not have an adverse effect on digestibility of nutrients.

ACKNOWLEDGEMENTS

This work was supported in part by the Ministry of Science and Technological Development of the Republic of Serbia (Project No. 31033 and Project No. 31081).

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EFFECT OF DIET ON LIPID CONTENT AND FATTY ACID PROFILE OF COMMON CARP (*Cyprinus carpio L.*)

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ABSTRACT: Production of fish in Serbia is insufficient despite of favorable climate properties. Dominant form of national aquaculture system is semi-intensive carp breeding. Except cereals, in the recent years extruded and pelleted feed are increasingly used for carp nutrition in Serbia. The aim of this study was to compare the lipid content and the fatty acid (FA) profile of carp (*Cyprinus carpio*) raised in carp fish farms with traditional breeding technology based on maize as well as complete extruded feed. Total lipids of fish fillets were extracted by accelerated solvent extraction (ASE) and FA profile was determined by gas-liquid chromatography equipped with flame ionization detector. Results were compared by multivariate data analysis. Principal Component Analysis (PCA) of the total FA profile clearly differentiated carp according to diet. Carp fed maize contained higher amount of total lipids (8.59%) compared to carp fed extruded feed (4.71%). Content of saturated fatty acids (SFA) of two groups of carp was similar. Carp fed extruded feed had lower content of monounsaturated fatty acids (MUFA) (42.43%) and higher content of polyunsaturated fatty acids (PUFA) (32.56%) than carp fed maize (61.78%, 12.99%, respectively). A higher content of n-6 (27.99%) and n-3 (4.57%) PUFA as well as better n-3/n-6 PUFA ratio (0.16) were found in carp fed extruded feed than in carp fed maize (11.75%, 1.24%, 0.10, respectively). Obtained results reveal that diet significantly influence the quality of carp flesh. Lipid content significantly decrease and PUFA content as well as n-3 to n-6 ratio increase when using extruded feed.
Key words: *carp, maize, extruded feed, fatty acids*

INTRODUCTION

Aquaculture is the fastest growing sector of food production in the world that provides more than half of the total amount of fish demand (FAO, 2010). Out of the total cyprinid production in the world, carp (*Cyprinus carpio*) accounted for 18% of the total production (Takeuchi et al., 2002).Carp is the most important warm-water fish in some European countries.

Production of fish in Serbia is insufficient despite of favorable climate properties. Dominant form of national aquaculture system is semi-intensive carp breeding. Semi intensive farming system is based on combination of natural food in the fishpond and additional food mostly simple grains such as wheat, corn and barley. In the last few years there have been taken measures, which might contribute to intensification of aquaculture by improving breeding technology.

Fish diet has a large influence on the chemical and fatty acid composition (Caballero et al., 2002, Tocher et al., 2004, Steffens and Wirth, 2007). The increased nutritional value of fish by using complete feed mixtures as fish diets enables to improve production quality and increase economic yields in fishpond (Markovic, 2010). Recent years, extruded and pelleted feed are increasingly used in carp nutrition in Serbia.

The aim of this study was to compare the lipid content and the fatty acid profile of carp (*Cyprinus carpio*) reared in carp fish farms with traditional breeding technology based on cereals and carp fed complete extruded feed. Investigation of the effect of diet on the lipid content and fatty acid composition of carp would contribute to the promotion of aquaculture and of the quality of carp flesh.

MATERIALS AND METHODS

Eight samples of two-year old carp were caught from two fishponds with semi-intensive breeding which are situated in the northern, lowland area of the country, in October 2009. Average weight of fish was 1900 g. In the first fish pond, maize was used as complementary feed (8.58% proteins, 4.5% lipids). In the second one fish was additionaly fed complete feed mixture consisting of maize, soybean meal and fish meal (23.81% proteins, 6.97% lipids).
Lipids of fish fillets for fatty acid determination were extracted with hexane/isopropanol mixture by accelerated solvent extraction (ASE 200, Dionex, Sunnyvale, CA), (Spirić et al., 2010). Solvent was removed under the stream of nitrogen at 50ºC until dryness. Total lipids were further converted to fatty acid methyl esters (FAMEs) by trimethylsulfonium hydroxide (EN ISO 5509:2000). FAMEs were determined by gas-liquid chromatography (GLC, Shimadzu 2010) equipped with flame ionization detector and capillary HP-88 column (length 100m, i.d. 0.25 mm, film thickness 0.20 μm). Injector and detector temperature were 250ºC and 280ºC, respectively. As carrier gas nitrogen was used at flow rate of 1.33 mL/min. The injector split ratio was set at 1:50. Total analysis time was 50.5 min. The chromatographic peaks in the samples were identified by comparing relative retention times of FAME peaks with peaks in Supelco 37 Component FAME mix standard (Supelco, Bellefonte, USA). Results were expressed as mass of fatty acid (g) in 100 g of fatty acids. Statistical analysis was carried out by applying principal component analysis (SAS Institute Inc. JMP 8.0.1 software).

RESULTS AND DISCUSSION

Fatty acid profile of carp fed maize and carp fed extruded feed from two semi-intensive rearing systems are presented in Table 1. Content of SFA, MUFA and PUFA as well as n-3 and n-6 PUFA and n-3/n-6 ratio was calculated.
Two groups of carp had similar content of SFA. Carp fed extruded feed had lower content of MUFA (42.43%) and higher content of PUFA (32.56%) than carp fed maize (61.78%, 12.99%, respectively). A higher content of n-6 (27.99%) and n-3 (4.57%) PUFA as well as better n-3/n-6 PUFA ratio (0.16) was found in carp fed extruded feed than in carp fed maize (11.75%, 1.24%, 0.10, respectively).
Carp fed maize contained higher amount of total lipids (8.59%) compared to carp fed complete extruded feed (4.71%). Steffens, 1997; Viola and Arieli 1983 found similar results for carp breed on supplementary maize feeding.

Fatty acid profile may be a useful tool for identifying fish according to ingested food. Comparison of fatty acids using multivariate data analysis (PCA) and linear regression have shown that the composition of fatty acids in flesh of fish fed different type of food is clearly distinguished and it is similar to fatty acids of their feed (Barrado, et al. 2003).
Principal Component Analysis (PCA) of the total FA profile (e.g. considering every FA as a variable) clearly differentiated carp according to diet (Figure 1 and 2). PCA of the fatty acid profile resulted in three principal components model describing 85.7% of the total data variability. The score plot of the first two principal components revealed grouping of samples in two distinct clusters along the first principal component direction (Figure 1 and 2).

Table 1. Fatty acid composition of carp (n=8) fed maize and carp fed extruded feed (% of total fatty acids) and total lipid content (%)

Fatty acid	Carp (maize) (n=8)	Carp (extruded feed) (n=8)
14:0	0.74±0.11	0.84±0.07
15:0	0.11±0.06	0.19±0.04
16:0	18.43±1.29	17.81±0.76
16:1	6.97±0.77	5.01±0.83
17:0	0.15±0.07	0.34±0.08
18:0	5.16±0.57	4.49±0.28
18:1cis-9	50.38±4.33	33.09±2.46
18:1cis-11	2.74±0.26	2.57±0.18
18:2n-6	10.05±2.19	26.09±1.81
18:3n-6	0.26±0.09	0.34±0.05
18:3n-3	0.80±0.54	2.23±0.27
20:1	1.69±0.32	1.76±0.24
20:2	0.33±0.07	0.68±0.09
20:3n-6	1.11±0.17	0.88±0.10
20:3n-3	0.06±0.09	0.48±0.11
22:1+20:4	0.65±0.29	1.35±0.28
20:5n-3	0.14±0.11	0.58±0.14
22:5 n-3	0.06±0.12	0.28±0.08
22:6n-3	0.18±0.19	1.01±0.27
SFA	24.58±1.29	23.66±0.80
MUFA	61.78±4.28	42.43±2.93
PUFA	12.99±3.03	32.56±2.37
n-3	1.24±0.99	4.57±0.66
n-6	11.75±2.30	27.99±1.91
n-3/n-6	0.10±0.07	0.16±0.02
Total lipids	8.59	4.71

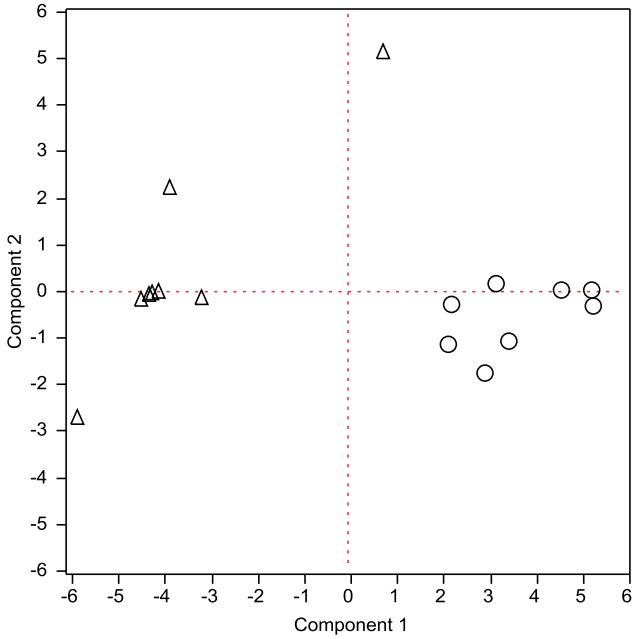

Figure 1. Principal component analysis score plot of the fatty acid profile of conventionally reared carp on maize (marked with triangles) and carp fed extruded feed (marked as circles)

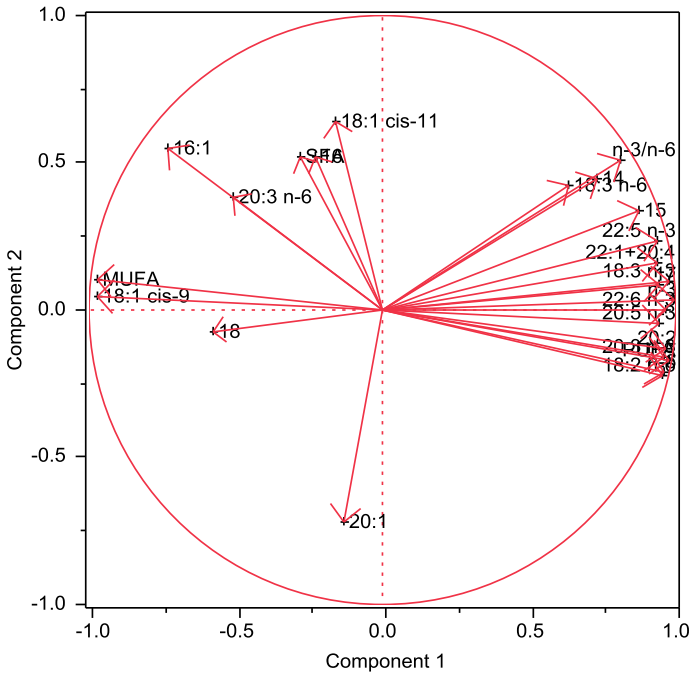

Figure 2. Principal component analysis loading plot of the fatty acid profile of conventionally reared carp on maize and carp fed extruded feed

Figure 2 indicate that two groups of carps with different feeding regime are clearly distinguished. Among fatty acids which mostly contribute to the variability on the negative part of the first principal component are C18:1cis-9, C16:1, C20:3n6, C18 and MUFA corresponding to carp fed maize which contains higher quantities of these fatty acids. Fatty acids which contribute to the positive part of the first component are C18:2n-6, C18:3n-3, C20:2, C20:3n-3, C20:5n-3, C22:5n-3, C22:6n-3 and PUFA corresponding to the groups of carp fed extruded feed. Thus, separations between two groups of carp may reflect differences in fish feed.

Research of Farkas et al.,1978 have shown that diet of carp rich in carbohydrates and poor in fats content (diet based on corn and wheat) increases of biosynthesis of C16 and C18:1 FA. Higher content of oleic acid in carp fed grains only resulted in a higher MUFA content, what is in accordance with results of Buchtova et al., 2007 and Fajmonova et al., 2003. Supplementary feeding of carp with maize resulted in lower content of n-3 and n-6 essentially FA in fish flesh. This is due to lower share of natural feed in the diet of carp that was supplementary fed grains (Steffens, 1997).

CONCLUSION

Obtained results reveal that diet significantly influence the quality of carp flesh. Fatty acids are essentialy important in human nutrition, particularly EPA and DHA. Quantities of these fatty acids and n-3/n-6 ratio are some of the indices of nutritional value of fish. Lipid content significantly decrease, PUFA content and n-3 to n-6 ratio increase by feeding carp extruded feed.

ACKNOWLEDGEMENT

This work was supported by the project TR-31075 funded by the Ministry of Education and Science of the Republic of Serbia.

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THE INFLUENCE OF FEED QUALITY AND FEEDING TECHNOLOGY ON THE DISEASE OCCURANCE AND ITS CONTROL

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ABSTRACT: In modern poultry farming, requirements regarding to nutrition and health care are more demanding because high productivity of poultry hybrids aproches its biological potentials. In the paper, the nutritional requirements and most frequent deviations are analyzed in detail, such as: the structure and distribution of feed, feeding space, expert and technical aberrations in composition of commplete diets. Proper and forehand elimination of factors that had lead to particular nutripathy, directly linked to feed or feeding technology, represent the key of successful poultry production.
Key words: *poultry, feed quality, feeding technology, disease, control*.

INTRODUCTION

Aiming for profitable food production that contains no harmful substances, high requirements for the new very demanding poultry lines must be provided (Kapetanov, 1997). High productivity that reaches close to the biological maximum of poultry implicates high requirements related to nutrition and health protection that are not easily acomplished. Thus in our poultry farming numerous problems occur with impact to the quality of final products and health status of birds (Kapetanov i sar., 1997). These problems are defined as nutripathy.
In the paper most frequent causes and consequences of nutripathies in poultry production are presented and means to recognize, reduce or eliminate them.

THE CHARACTERISTICS OF FEED TYPES

Feed granulation. Poultry is natural seed-eater. Roughly mashed diets are used in chickens and adult poultry. If the content of very small particles is high or the granulation is too large, selective intake of feed and certain components may occur (Pavkov i sar., 1993). During the warm months when ventilation system is on the forced regimen, too many fine, powder-like particles containing essential nutrients are pooled out. White dust cover surfaces in front and around the outlets including roof and parts of farm surrounding.
The type of diet differs because the meal can be provided as mashed, pelleted or extruded. In some regions of the world it is common to mix whole grains in complete diets before feeding. In general, pelleted or extruded feed is easier to handle in compair to mashed one. It is known that single particles should not extend 3 mm in diameter in chick starter and 5 mm in grower diets.

The size and hardness of pellets. The size and hardness of pellets must be adjusted to the poultry species, age and category. However, large and hard pellets are not so rarely found. This may be evident immediatelly after the first body weight measuring that tend toward rapid growth decrease. On clinical examination small number of birds is seen around the feeders throwing out the pellets in search for adequate by size and hardness. The rest of the flock follow man consistently pecking the footware. The significant amount of feed is located around the feeders but sometimes it is necesary to remove the superficial litter to discover it. In case of prolonged consumption mechanical damage in oral cavity may occur. From our

experience such cases were often seen in fattening turkeys. It is possible to test the pellets by disolving them in a glass of warm water, that has to take no longer than five minutes.

Feeding with whole grain in mating period. It is observed that many peacocks die during the mating period after consumption of whole corn seed. In the spring, males produce very loud sounds to atract the females when they find feed. Females respond to the call and the male swallow the whole seed of corn, which sometimes ends in trachea („the jewish end"). For several hours males try to take out the corn by moving backward with their neck outstreched. In time the corn swell up causing dyspnoe and death. This is why males should not be fed with whole grain, especially corn in the mating period.

FEEDING SPACE AND FEED DISTRIBUTION

The optimal feeding space is essential in any type of feeding. Insufficient feeding space influence reduced weight gain and threaten flock uniformity. Nowadays, in the first ten days of life in broiler production biodegradable paper is used (chick paper) as additional feeder; it is wide 1 to 1.5 meters and the length is one third of the building. This method provides enough quantity of complete diet but also its availability. Often chicks „take a nap" in feeders thus reducing the overall feeding space, especially during the colder months.
Insufficient feeding space and poor feed distribution in growing flocks of layers or parents, that include feed restriction may cause health problems and poor production. The decrease of growth may occur, inadequate flock uniformity and high variation interval. Because the birds compete over the feed, they can get injuries, most frequently on head, necks and legs. Between 8 and 15 weeks of age, swollen joints on one or both legs are often seen and infection with *Staphylococcus aureus* (Kapetanov i sar., 1999). Mechanical injuries are mostly seen in heavy line males, sometimes causing the lower reproductive ability in later, mature age. Frequently birds compensate reduced feed consumption by feeding with litter, particularly fresh straw or wood shaving. In such cases clostridiosis was diagnosed on numerous occasions (Kapetanov i sar., 2008).
In growing flocks in which anticoccidials are used to prevent coccidiosis instead of vaccination, sometimes feed restriction is over seen. The clinical coccidiosis is very common in such flocks, particularly if feeding technology is not proper. In our cases, mixed infections, coccidiosis and clostridiosis was quite often seen.
In layers, kept in cages during the production feeding system becomes deformed over time, causing uneaven distribution of meal.
Insufficient feeding space and inadequate distribution of feed cause the increased mortality. In the structure of mortality dominate: suffocation, „hungry mortality", technological discard, mechanical injuries and its consequences (Kapetanov i sar., 1997; Kapetanov i sar., 2000; Kapetanov i sar., 2003; Orlić i sar, 2005; Živkov-Baloš, 2007).

TECHNICAL ABBERATIONS IN COMPLETE DIETS

Drug toxicity. When certain therapy is indicated precausion must be taken in flocks fed with complete diets that contain coccidiostats. The simultaneous use of some drugs, for example ionophore antibiotics and tiamulin, monensin and suplphonamides or erithromycine etc., favor its toxicity even in therapeutical dose. Side effects are expressed in short time with excitation, frequent and hard breathing, swollen head, exudative and haemorrhagic diathesis and death in position with streched neck and legs. Thus every shippment of feed must have declaration in which added substances and contraindications must be documented.

Calcium deficiency. The consequences of calcium deficiency in complete diets were found in all production stages, however the most prominent and detrimental effects were during the peak of egg production. After viral infections are excluded, it is diagnosed easily at this production phase since large number of cracked eggs and soft egg shell are found. However,

if pre-lay rations have less protein and calcium content, the diagnose is more difficult. In our experience clinical features in layers include lie with head put down, ruffled neck feathers, sometimes streched legs and pinguine position, moving with the help of wings, softened and deformed beak and deformities on brest region caused by flexure of keel, ribs and spine.

Deficiency of vitamine E. Low content of vitamine E in poultry rations induce two syndromes, that can occur solitary or together, encephalomalathia and exudative diathesis. Cases of encephalomalatia are mainly seen at the age of 2 to 4 weeks, sometimes earlier at 7 days, with clinical signs of: drowse, ataxia, movements in circle, tremor, uncontrolled shiverring, torticolis. The death is usually in side position. On postmortem examination cerebral oedema and solitar petechial haemorrhagies are present, also possible subcutaneous oedema on neck, chest and abdomen and cold and blue colouration of adjecent skin. From our experience, cases of deficiency occur during the summer in broiler flocks with exellent weight gain. The incriminated feed in cases of exudative diathesis had specific aroma like unsaturated fat**,** particularly pronounced after it was rubbed between palms. The successful therapy was application of vitamine E and selenium, for example 50 ml per 1000 kg body weight of commercial preparation Evitaselen, for three days. Also multivitamine preparations containing high vitamine E and A gave good results.

Intoxication with inadequately heat treated soy products. Besides high valuable nutrient content, soy bean has some undesireble and toxic substances. Crude soy beans contain tripsin inhibitors, lipid oxidase, hemagglutines and allergenes. All these substances are proteins that are denaturated during heat treatment and thus decrease their activity to the harmless level. In our two year investigations significant percentage of soy bean products was under or too much heat treated: 19.56% of 19,56% soy bean meal, 28.95% of extruded soy bean meal and 14.29% of soy cake. Intoxication with unsufficiently treated soy bean products was determined mostly in broilers, because they use such nutrients at most. The clinical signs were evident usually at the age of 3 to 4 weeks, including: impaired uniformity, low vitality and grouping around the heaters, paresis and paralysis and dysrupted behaviour with the pecking of litter, wall, feeders and drinkers. Wet litter was observed because of yellow to pale redish diarhoea and undigested feed particles. The weight gain was poor. Based on case history, clinical signs and section findings of catarrhal enteritis and underdeveloped gizzard, the chemical analysis of soy products was advised. Substitution with safe product and treatment with multivitamine preparations with essential amino acids provided good results (Kapetanov i sar., 2010).

The use of freshly harvested grains in complete rations. Feed producers and farmers often use freshly harvested grains like wheat or corn, because of shortage or high market prices. The moisture content and histamin level are too high in these grains. Shortly after consumption, clinical ilness begins, with symptoms of paresis, paralysis, exudative diathesis, protruding diarrhea with significant amount of undigested particles, catarrhal enteritis. Combined treatment with vitamine E and selenium was beneficial and the problem was eliminated. Such cases were most frequently observed in broilers.

CONCLUSIONS

The adequate and forehand removal of condition that caused certain nutripathy linked to feed or feeding technology, represent the key for successful poultry production.

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THE IMPORTANCE OF BROKEN CORN KERNELS EXTRUSION

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ABSTRACT: The feed industry has a target to produce variety feed that fully satisfy the nutritional needs of certain types and categories of animals. Complete feed meals for animal feeding have to fully meet the nutritional needs of certain types and categories of animals. The energy level of a feed meal can be achieved by adding appropriate amounts of fat and grains. In the production of feed, in relation to other cereals, corn is the leader due to its high energy (about 16 MJ/kg) and starch and low fiber content. In comparison to other cereals, corn is attributed with the best digestibility and taste. Before drying and storage, in the technological process of either trade or seed corn cleaning, significant quantity of broken grains is separated. Complete feed meals for animal feeding and feed have to fully satisfy Regulations concerning hygiene and quality. Improving hygiene and nutritive value of broken corn grains can be achieved by extrusion. In this research, broken corn grains were processed in the single screw extruder at a temperature of 90ºC. Total count and total number of yeasts and molds decreased from 270.000 to 12.000 and from 540.000 to 10.000 cfu/g, respectively. Due to extrusion process, certain physico-chemical changes occurred in raw material, primarily manifested in structural changes of starch, which is reflected in the increase in sucrose (from 2.28% to 5.19%) and reducing sugars (3.36% to 5.88%) content in extrudates.
Key words: *broken corn grains, extrusion, hygiene*

INTRODUCTION

According to the Directive of the European Union no. 178/2002 (Article 3, paragraph 4) "Feed for the animals is each component or product, including additives, whether processed, partially processed or unprocessed, intended for oral feeding of animals." For the modern intensive livestock production mixtures of different concentrated feed with supplements that have nutritive or some other significance are of particular importance. These compounds are being produced in accordance with contemporary standards, and meet the high demands of modern species and hybrids. Maximum homogenization of certain nutrients and supplements is experienced in modern plants, equipped with the most modern technology controlled by computer (Djordjevic and Dinić, 2011).

Complete feed for animals must fully meet the nutritional needs of a particular type and category of animals as well as the energy demand. The energy levels of compounds can be achieved by adding appropriate amounts of cereals and fats. In the production of feed, corn has a leading position compared to other cereals. The reason is its high energy content (16.2 MJ/kg), high content of starch, and relatively great amounts of fat and low fiber (Bekrić, 1999). Beside the best digestibility, corn also has the best taste in comparison to other cereals. Complete feed mixes for animals as well as feed must be hygienically safe and completely in accordance with the requirements of the Regulations on the quality of feed (2010). Before the drying and storing of corn, significant amounts of broken kernels are separated. Broken kernels are susceptible to contamination of molds that produce aflatoxin, which have a negative impact on growth and development of animals. In case microbiologically contaminated corn is used as a component of feed hygienically incorrect mixture that is a major threat to animal health is obtained.

Improvement of hygienic, nutritional, physical and chemical characteristics and viability of corn broken kernels can be achieved by extrusion process. This procedure contributes to: increased nutritive value of some nutrients, improved sensory properties (increased "sweetness" in the processed corn), microbiologically safe products (Jansen, 1991; Verheul, 1997) but also may inactivate possibly present thermo labile antinutrients. Extrusion is a heat treatment that includes high temperature/short time (HT/ST) principle of extrusion cooking, i.e. process in which the material is short time exposed to the high temperatures (Filipovic et al., 2004).
In this paper the technological parameters for extrusion process, physical-chemical characteristics and microbiology of broken corn kernels before and after extrusion are presented.

MATERIAL AND METHODS

Broken corn kernels, separated in the process of corn cleaning prior to artificial drying are used in the experiment. Before extrusion, moisture content of material should be adjusted to approximately 18%. That is a prerequisite for controlled extrusion temperature regime (Filipovic et al., 2008). Before extrusion corn broken kernels are grinded in a hammer mill with screen openings Ø4mm and moisture content was 18,74%. Extrusion is performed on the extruder with three heating segments, capacity of 150-170 kg/h extrudates. Installed electric power of extruder is 22 kW. Extrusion is performed at a temperature of 90°C. Extruded broken corn kernels are cooled in the vertical cooler in the intensive air stream generated by the fan.
The basic chemical composition (moisture, crude protein, crude fiber, crude fat and ash) and starch content in the starting raw materials and extrudates are determined by the methods of the Regulations on sampling methods and methods of physical, chemical and microbiological analysis of animal feed (1987). Total sugar content was determined by the Regulations on the physical and chemical methods of analysis for quality control of grain, milling and bakery products, pasta and deep frozen doughs (1988).
The total number of microorganisms is determined by the method of BS EN ISO 4833:2008, *Coagulase positive staphylococci* by method EN ISO 6888-1:2008, *Clostridium perfringens* by BS EN ISO 7937:2008, *Salmonella* spp by method EN ISO 6579:2008, the total number of yeasts and molds by method ISO 21527-2 and *Sulphite-reducing clostridia* by 15213:2003 ISO method.

RESULTS AND DISCUSSION

Chemical composition of broken corn kernels prior and after extrusion is presented in Table 1.

Table 1. Chemical compositin of broken corn and extruded broken corn kernels

Quality parameters	Broken corn kernels		Extruded broken corn kernels	
	% in sample	% in DM*	% in sample	% in DM*
Moisture content	18,74	-	14,45	-
Crude protein content	7,40	9,11	7,48	8,74
Fat content	3,37	4,15	2,76	3,23
Crude celullose content	3,17	3,90	2,62	3,06
Ash content	1,58	1,94	1,57	1,84
Starch content	61,17	75,28	61,19	71,53
Total sugar content	2,16	2,66	5,18	6,05

*DM-dry matter

Energy feed is produced by extrusion of broken corn kernels. Extrusion process significantly reduces the moisture content of tempered raw material (from 18.74% to 14.45%), which is

very important in terms of storage and keeping quality of extrudates. This fact is confirmed by the earlier results of Filipovic et al. (2005) and Filipovic et al. (2008).
In the extrusion process essential changes are occurring in starch complex, which is manifested in the increase of total sugars (from 2.66% in the d.m. 6.06%), reduction of starch content (from 75.28% to 71.52% d.m.) parallel to significant change in organoleptic properties. Extrusion process leads to changes in the carbohydrate complex of corn, i.e. decrease in starch content due to its degradation to dextrins and the total sugars increase. These changes bring about an increase *in vitro* and *in vivo* digestibility of starch, since starch gelatinization provides increased availability of enzymes to starch, and parallel to the above, leads to inactivation of the amylase inhibitor. Related to crude protein and minerals in broken corn kernels prior and after extrusion changes in the quantity are not registered. Similar conclusions are met by Kormanjoš et al. (2007) in studies on the impact of extrusion on hygiene of enriched corn meal. Table 2 presents the results of microbiology of broken corn kernels prior and after extrusion

Table 2. Content of microorganisms in the broken corn kernels prior and after extrusion

Microorganism	Broken corn kernels	Extruded broken corn kernels
Total number of microorganisms	270 000 cfu/g	12 000 cfu/g
Coagulase positive staphylococci	< 100 cfu/g	< 100 cfu/g
Clostridium perfringens	< 10 cfu/g	< 10 cfu/g
Salmonella spp	not detected in 50 g	not detected in 50 g
Total number of yeasts and molds	540 000 cfu/g	10 000 cfu/g
Sulphite-reducing clostridia	< 10 cfu/g	< 10 cfu/g

Due to the increased total number of yeasts and molds (540 000 cfu/g) broken corn kernels do not meet the requirements of the Regulations on the quality of feed (2010). Extruded broken corn kernels in relation to the starting raw material have significantly reduced total number of yeasts and molds (10 000 cfu/g) as well as total count of bacterial (12 000 cfu/g). This reduction was confirmed by Kormanjoš et al. (2008), in the process of corn grain extrusion. According to the Regulations on the quality of feed, broken kernels after extrusion are hygienically safe and can be used as a component in the preparation either complete or complementary animal feed.
Despite a relatively low temperature (90ºC)and a very short time (6-10 sec) of extrusion process, it is positively contributing to the reduction of microorganisms. Very high pressure of extrusion ranging from 30-40 bar, may explain the reduction total number of microorganisms.

CONCLUSIONS

- ✓ Extruded broken corn kernels is highly valuable energy feed that can be used for the preparation of animal feed
- ✓ In the extrusion process of major changes are occurring in starch complex. Increases the total sugar content in the extrudate is leading to the reduction in starch content.
- ✓ The process of broken corn kernels extrusion contributes to the reduction in the number of microorganisms thus providing a hygiene safe nutrient with long shelf-life.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III046012 financially supported by the Ministry of Education and Science, Republic of Serbia.

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PRESENCE OF CONSTITUENTS OF ANIMAL ORIGIN IN FEED

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ABSTRACT: Feed monitoring for the presence of ingredients of animal origin is performed in order to prevent transmissible spongiform encephalopathies. When it was found that the source of infection was feed, i.e. infectious ruminant protein processed in meat and bone meal (MBM), legislation which prevented these nutrients to enter a food chain was introduced all around Europe. Today in Serbia, as well as in EU, usage of MBM is completely banned for all farm animals. But, until 2011 presence of these nutrients had been officially banned just in the diets for ruminants. Such a partial limitation in Serbia was the reason for more frequent cross contamination of feed for ruminants with prohibited ingredients. Therefore, feed producers were obligated to separate lines for feed for ruminants, or otherwise, not to use MBM and fish meal. Control of such regulations has been carried out by laboratory testing of feed for ruminants, as well as feed for pigs and poultry, for the presence of constituents of animal origin by classical microscopy (Regulation (EC) No 152/2009 Annex VI). The most examined was feed for different categories of cattle. During year 2010 among 162 of these feed samples it was found 3.09% of positives, while the result for 2011 (1.85%) represents an improvement. Comparing the latest results to those from previous years we can conclude that better compliance to European standards is obvious in Serbian feed industry.
Key words: *feed, MBM, microscopy*

INTRODUCTION

After bovine spongiform encephalopathy (BSE) was first diagnosed, it was soon determined that this disease was transmitted through feed, i.e. through infectious ruminant protein processed in meat and bone meal (MBM). Eradication process had immediately started and one of the most important measures was the establishment of legislation which prevented these nutrients to enter the food chain (WHO, 2002).

In the European Union, according to Regulation 999/2001 and Regulation 1234/2003, use of processed animal proteins, including different types of MBM, is prohibited for all farm animals entering the food chain, excluding fish meal for nonruminants. Such a measure was taken in accordance to the fact that these nutrients represent a potential source of infection not only for animals, but also indirectly, through food of animal origin, for the people. Due to this strict approach epizootiological situation today is much better in Europe what shows that applied "BSE management" was mostly successful (Paisley et al., 2008).

Control system in Serbia, compared with the European Union, was more complex, due to the differences in legislation and applied preventive measures. In 2001 (Pravilnik, Sl.list 38/2001) feedstuffs of animal origin for the first time were officially banned in the diet for ruminants, but their presence was still allowed in the mixtures for monogastric animals. Identical measures were also prescribed by next Regulation (Pravilnik, Sl. glasnik 4/2010) which came into force in May 2010. However, as a final step in approaching EU legislation, the latest Regulation which came into force in April 2011 brought to Serbia identical feed ban as in the EU countries (Pravilnik, Sl. glasnik 96/2010).

Previous partial restriction on the use of feedstuffs of animal origin, on the one hand reduced the negative economic consequences of their complete elimination and kept all the benefits of these nutrients to animal production and performances, but on the other hand, the possibility of cross contamination of feed intended for ruminants by prohibited ingredients, coming from feed for monogastric animals, was largely opened. For this reason, feed manufacturers were obligated by the Veterinary law Art. 110 (Zakon o veterinarstvu, Sl.

glasnik 91/05) to separate the special line for the production of feed for ruminants, or otherwise, to eliminate animal feedstuffs from all rations completely. Control of these conditions was carried out as part of a special program of the Veterinary Directorate and in accordance to Directive for preventing, detecting and eradication of transmissible spongiform encephalopathies (Naredba, Sl. glasnik 17/2006). The laboratory testing of feed for different animal species for the presence of constituents of animal origin (MBM and fish meal) has been done by conventional microscopy according to the official EU procedure.
The aim of this study was to compare the results obtained over the last two years, especially bearing in mind the change in regulation that occurred last year. From this point it is necessary to assess the effectiveness of new measures.

MATERIAL AND METHODS

In order to assess the success of applied regulations, we compared the results of microscopic analysis of 251 feed samples for cattle, sheep, goats, pigs and poultry in 2010 and 310 samples in 2011. All examinations for the presence of ingredients of animal origin were carried out using conventional microscopy, ISO 17025 accredited method in the Institute of Veterinary Medicine of Serbia, which was applied in accordance to Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, Annex VI.

RESULTS AND DISCUSSION

During 2010 total of 251 samples of feed mixtures for different animal species were tested by classical microscopy. Most of them were feed samples for cattle - 162 in total. Among them 3.09% were found positive for the presence of particles of animal origin (MBM or fish meal), what made them unusable as intended, because they did not correspond to Article 51 of the Regulation for the quality of animal feed (Pravilnik, Sl. glasnik, 4/2010), as well as to the Regulation on the establishment of measures for early detection and diagnosis of infectious diseases transmissible spongiform encephalopathies, methods of their implementation, as well as measures to prevent the spread, prevention and eradication of infectious diseases (Pravilnik, Sl. glasnik 96/2010).

Table 1. Results of microscopy testing in 2010

	cattle	sheep	goats	pigs and poultry
TOTAL	162	8	6	75
Negatives	157	8	5	69
Positives	5	0	1	6
% of positives	3.09	0	16.67	8

Feed samples for sheep did not contain any constituents of animal origin, while one among six samples of mixtures for goats was contaminated. Feed for pigs and poultry was fortified mostly with fish meal and percentage of such samples averaged around 8%.
Total of 310 feed samples for cattle, sheep, goats, pigs and poultry were examined in 2011. Among 162 samples of feed for cattle only 3 positive cases were found, what was significantly lower percentage – 1.85% comparing to previous year. No contamination was detected in feed for other ruminants, while presence of feedingstuffs of animal origin in mixtures for pigs and poultry was at the similar level – 8.33% and also mainly derived from fish.

Table 2. Results of microscopy testing in 2011

	cattle	sheep	goats	pigs and poultry
TOTAL	162	15	1	132
Negatives	159	15	1	121
Positives	3	0	0	11
% of positives	1.85	0	0	8.33

Comparing the results of monitoring of mixtures for different categories of the most common animal species bred in Serbia, obtained during the period 2010 - 2011, a significant improvement in the presence of prohibited components of animal origin could be observed (Table 1 and 2). Actually, the lowest percentage of positive samples in feed for all ruminants was found in 2011, while presence of animal particles in samples for monogastric animals stayed at the similar level and still corresponded to regulations, as those were usually fish meal ingredients. Such downward trend is expected to continue also during this year, especially having on mind that new regulation, which brought complete MBM ban for all farm animals in Serbia, came into the force in April 2011. That means that risk of cross contamination has become extremely reduced since that time.
Such a favorable situation and the obvious improvement can be interpreted as a consequence of successful harmonization with EU legislation by introducing several new regulations in Serbia: Veterinary Law (Zakon o veterinarstvu, Sl. glasnik RS 91/05) and the Law on Food Safety (Zakon o bezbednosti hrane, Sl. glasnik RS 41/09), as well as a number of other regulations which contribute to better control and progress in the food and feed industry. This also proves the success of BSE risk management which has been established in the EU and confirms positive results of European steps that we follow (Paisley et al., 2008).

CONCLUSIONS

Based on the results of microscopic examination of feed for cattle, sheep, goats, pigs and poultry for the presence of constituents of animal origin it could be concluded that in recent years there has been a general improvement of the production quality in our feed industry. In many cases the highest European standards have been applied, what makes our manufacturers competitive on foreign markets. On the other hand, laboratory analysts and other professionals close to those profiles, by better future engagement and pragmatic cooperation in this area, can contribute to further progress in the safe food production.

ACKNOWLEDGEMENTS

This paper has been published thanks to project III 46009 “Improvement and development of hygienic and technological processes in the production of food of animal origin in order to obtain high-quality and safe products competitive on world markets“ financed by the Ministry of Education and Science of Republic of Serbia.

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FEED AS CAUSE OF ASPERGILLOSIS IN FLOCK OF TURKEY POULTS

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ABSTRACT: Aspergillosis is frequent fungal disease of different avian and mammal species, caused by fungi of genus *Aspergillus*. The disease is characterized by inflammatory changes in the respiratory system and sometimes has generalized onset when more organ systems are affected. These fungi are well known aflatoxin producers which, besides other microorganisms, can also be found in feed raw materials. However, the presence of fungi is not a proof of mycotoxins contamination, because they are produced in specific conditions. In this paper are presented results of examination of a flock of turkey poults, 21 days old, at one farm in Serbia. Clinical signs of central nervous system disorder were observed. After necropsy, in ten out of twelve necropsied turkey poults multiple yellowish-white granulomas on lungs were found. In nine out of twelve necropsied turkey poults solitary yellowish-white granuloma on sagital section of the cerebrum or cerebellum were found. Mycological finding revealed fungi *Aspergillus fumigatus*. Two stain methods were used: haematoxylin-eosin (HE) and Grocott methenamine silver (GMS). Histopathological analysys of lung and brain has revealed the presence of granulomatous foci and caseous necrosis with surrounding region of proliferation including giant cells, macrophages, heterophils and lymphocytes and outer capsule of connective tissue. The fungal hyphae were hardly visible or not in HE stained sections, while septed and arborized hyphae were easily demonstrated by GMS method predominantly in central parts of granuloma. Mycological examination of feed showed the presence of *Aspergillus fumigatus and Aspergillus flavus*, as well as ELISA detectable level of aflatoxins (3.00 µg/kg).
Key words: *Aspergillus, aflatoxin, turkey poults, granuloma*

INTRODUCTION

Aspergillosis is frequent, economically important, fungal disease of different avian and mammal species caused by fungi of the genus *Aspergillus*. The disease is characterized by inflammatory changes in the respiratory system and sometimes has generalized onset when more organ systems are affected. Clinical signs of aspergillosis depend upon which organ or system organ are involved and whether infection is localized or generalized. Pathomorphological substrate of this disease is mycotic granuloma (*aspergiloma*) which is commonly found in lungs and air sacs. Numerous reports have described encephalitic or meningoencephalitic aspergillosis in turkey poults, ducklings and chickens (Knežević and Matejić, 1996; Ivetić et al., 2003; Ozmen and Dorrestein, 2004; Saif, 2008; Singh et al., 2009, Kureljušić et al., 2011). For diagnostic of mycotic infection is necessary to use more methods included clinical examination of flock, macroscopic examination of carcasses, isolation of pathogens, as well as histochemical methods for detection tissue lesions and fungal elements. Histochemical methods which can be used are routine hematoxylin-eosin staining method, periodic acid-Schiff (PAS) and specific staining method for fungi Grocott methenamine silver method (GMS) (Ozmen and Dorrestein, 2004).

Although detrimental effects of mouldy food and feed have been well known for a long time, *Aspergillus* fungi and their metabolites called aflatoxins were not determined until the 1960's. A few hundred mycotoxins have been identified so far, but only a fraction of these are considered harmful to humans (Riley, 1998). Additionally, 20-30 mycotoxins have medical, nutritional, ecological and economic significance, due to their incidence and toxicity,.

According to the target tissue aflatoxins are hepatotoxins which represent a mixture of several chemical substances. They are heterocyclic secondary metabolites of *Aspergillus* fungi. Their synthesis depends on environmental conditions in which fungi grow, but their production is also dependant on the fungus strain (Pasteiner, 1998). Although it is well known that aflatoxins are metabolites of *Aspergillus flavus, Aspergillus parasiticus* and more recently *Aspergillus nomius* (Rodrigues et al., 2007), there are certain strains of these fungi which are not toxin producers at all. Fungi produce aflatoxins in conditions of high moisture content, temperature and adequate substrate. Synthesis is highest when humidity is above 13% and temperature is between 24^{o}C and 37^{o}C. That is why warm and wet geographic regions are the most favorable environments for aflatoxins. *Aspergillus flavus* is the main producer of the well known carcinogenic aflatoxins. It is a fungus widely spread in nature which can be found in different cereals and many other feedingstuffs. It is particularly typical for peanut which is infested underground, especially in tropical and subtropical regions.
According to its frequency of occurrence and its toxicity aflatoxin B_1 is the most important among all in the group (Beuchat, 1987). Aflatoxicosis is manifested by different pathomorphological changes, especially in the liver, but also in kidneys, cardiovascular and nervous system. Moreover, it exerts detrimental effects on performances, primarily in broiler and layer production, but also in swine breeding and all other modes of animal husbandry. Residues can be found in the liver, muscles, guts, kidneys and fatty tissue, as well as in meat, milk and eggs of animals which ingested contaminated feed (Resanović and Sinovec, 2005).
Besides clinical and patomorphological manifestation of aspergillosis in turkey poults, this paper also presents results of mycological and mycotoxicological examination of feed which, as well known, is very often contaminated with moulds.

MATERIAL AND METHODS

In this paper, we examined the carcasses of 12 turkey poults, three weeks old which originated from one farm in Serbia. The mortality rate in the flock was 7,2%. Clinical examination of the flock revealed moderate depression of many turkey poults and nervous symptoms which were expressed in the form of ataxia, torticollis, paresis and paralysis of legs and wings were observed. Food consumption was reduced leading to reduced weight gain. After necropsy, for histopathological and mycological examination samples of lung and brain tissue were taken. Samples for histopathological analysys were fixed in 10% buffered formalin, routinely processed and embedded in paraffin blocks. Paraffin sections about 5 μm were stained with hematoxylin-eosin (HE) and Grocott methenamine silver (GMS) methods. Samples for mycological examination were inoculated onto *Sabouraud dextrose agar* and incubated at temperature of 25^{o}C under aerobic conditions for isolation of infectious agents. To avoid bacterial contamination in the substrate was added 20 IU/ml penicillin G and 40 mg/ml streptomycin sulfate. Macroscopic and microscopic examination of colonies were performed according to Quinn et al. (2002). Samples of feed and litter were also taken for mycological analysys.
Mycological analyses of feed samples were done with the aim to identify the level of contamination with fungi of *Aspergillus* genus and also to detect the presence and level of contamination with their metabolites aflatoxins.
Samples were tested in accordance to ISO 21527-2:2008 procedure. Presence of aflatoxins was detected using ELISA method as directed in the instructions of commercial kit (Neogen). Results were compared and interpreted according to the national regulations (Pravilnik, Sl. glasnik RS, br. 4/2010).

RESULTS AND DISCUSSION

External examination of turkey poults carcasses indicates less feathering of the skin especially in the region of chest. In ten out of twelve necropsied turkey poults granulomatous pneumonia with multiple yellowish-white granulomas, one to three millimeters in diameter on lungs were found. In two out of twelve cases granulomas were found on the cranial part of the cerebrum, and in one case granuloma was situated on central part of cerebellum. Granulomas were solitary, yellowish-white in colour, three to five millimeters in diameter, and on sagital section of the cerebrum or cerebellum were found (Figure 1).

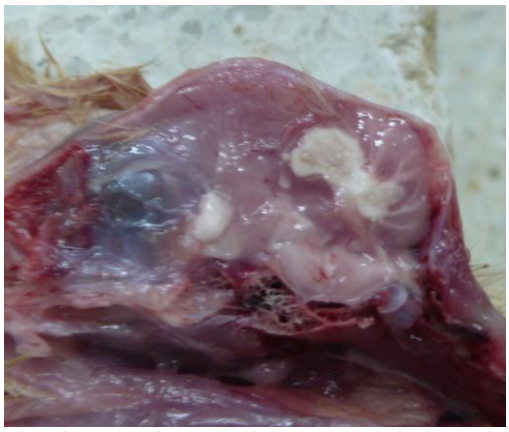

Figure 1. Brain of turkey poult, granuloma on sagital section of cerebellum

Data from litarature indicate that aspergilous granulomas (aspergilomas) usually occur in the lungs and air sacs although there are data on the occurrence of aspergilomas in the cerebellum, just for turkey poults (Schulz, 1991; Ozmen and Dorrestein, 2004; Saif 2008, Kureljušić et all, 2011). Interestingly, the granulomas occur either in cerebrum or the cerebellum (Saif, 2008), which is confirmed in this case. Rarely, aspergillomas occur simultaneously in both parts of brain (Saif, 2008). In addition to this finding, in all necropsied turkey poults hyperemia of brain blood vessels were found. In other organs there were no macroscopically visible changes.

Mycological finding revealed fungi *Aspergillus fumigatus*. Growth in the medium was observed after 24 hours, and after four days, grown colonies were white and about 2 cm in diameter. By the seventh day the colony diameter increased to 3.5 cm, and there was a color change in the central part of the colonies from blue-green to the gray-green, while the edges remained white (Figure 2). Based on macroscopic and microscopic characteristics of colonies, the culture was identified as *Aspergillus fumigatus.*

Figure 2. Growth of Aspergillus fumigatus on *Sabouraud dextrose agar*

For the evaluation of histopathological changes and demonstration of fungal hyphae, two stain methods were used: haematoxylin-eosin (HE) and Grocott methenamine silver method.

Histological finding in the lung and in the brain was the same. Granulomatous reactions with central caseous necrosis were observed in the HE stained slides. There was surrounding region of proliferation including giant cells, macrophages, heterophils and lymphocytes and outer capsule of connective tissue (Figure 3, Figure 4).

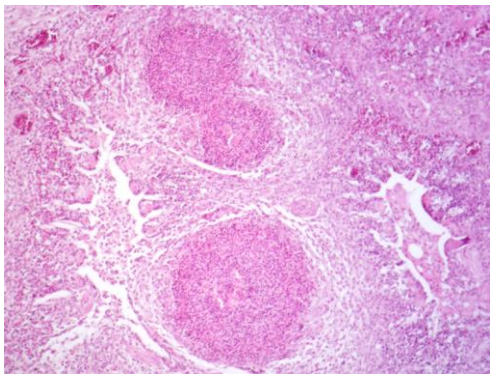

Figure 3. Lung, turkey poult, multifocal granulomatous pneumonia, HE, X200

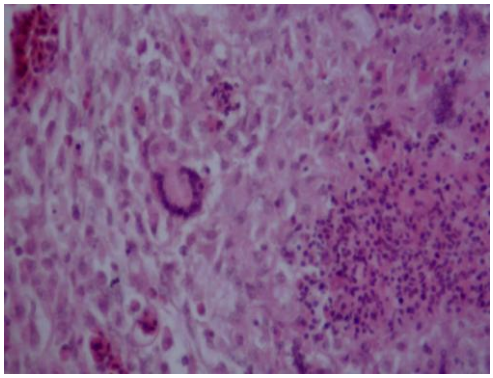

Figure 4. Lung, turkey poult, infiltrate of granuloma consist of giant cells, macrophages, heterophils and lymphocytes, HE, X400

The fungal hyphae were hardly visible or not in HE slides, while septed and arborized hyphae were easily demonstrated in all samples stained by Grocott methenamine silver method predominantly in central parts of granuloma (Figure 5).

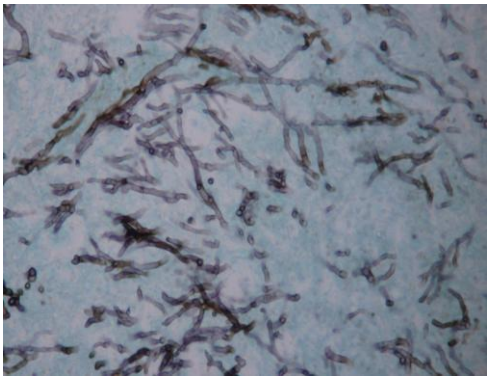

Figure 5. Brain, turkey poult, arborized and septed hyphae, GMS, X400

In some cases, in the sections of brain and lung stained by HE method, granulomatous changes were characterized only by presence of smaller or larger number of necrotic foci, which are infiltrated with macrophages, giant cells and heterophil granulocytes. These changes are described in the literature as early initial lesions (Jensen et al., 1997). In three out of twelve cases in HE stained sections fungal hyphae were not apparent. Grocott methenamine silver method in all twelve cases revealed septed and arborized hyphae.

Grocott methenamine silver method is described in the literature as a very reliable method for direct visualization of the different fungal elements in tissues (Ozmen and Dorrestein, 2004; Singh et al., 2009).
Mycological examination of feed showed the presence of *Aspergillus fumigatus and A. flavus*, as well as certain level of aflatoxin - 3.00 µg/kg. Although this level did not exceed limit layed by actual legislation, having on mind cumulative effects and possibal chronical exposure to the harmful influence, as well as indirect loses due to drop of performances and production and occurence of secondary infections, even such amounts must not be neglected. Mycological analysys of litter revealed also *Aspergillus fumigatus*. It is known from literature that contaminated litter and feed are often the source of *Aspergillus fumigatus* (Saif 2008).

CONCLUSIONS

Microscopic finding of typical mycotic granulomas in the lung and brain with central caseous necrosis and fungal hyphae surrounded by macrophages, giant cells, lymphocytes, heterophils and capsule of connective tissue pointed to aspergillosis. For diagnostic of mycotic infection is necessary to use more methods included clinical examination of flock, macroscopic examination of carcasses, isolation of pathogens, as well as histochemical methods for detection tissue lesions and fungal elements. Beside aspergillosis in turkey poults, in this case *Aspergillus fumigatus and A. flavus*, as well as certain level of aflatoxin - 3.00 µg/kg were confirmed in the feed. However, in the case of lower levels of mycotoxins still remains the risk of rigid interpretation of the regulations, as it is well known that the use of feed with low content of mycotoxins in the longer time period shows similar effects as short-term use of feed with higher amount of mycotoxins. The problem is getting more complicated as the interaction of mycotoxins present in feed increases harmful effects. Therefore, the assessment of utilization of feed should be depend on the institutions and experts that deal with this problem as one of the special scientific and professional activities. Also, on the basis of everything above, it can be concluded that solving these problems requires a multidisciplinary team approach.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III 46009 financed by the Ministry of Education and Science, Republic of Serbia.

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IMPORTANCE OF ZOOPLANKTON AS LIVE FEED FOR CARP LARVAE

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ABSTRACT: Zooplankton is a very important source of natural food for larvae of many aquaculture species. In semi-intensive systems, which are the dominant type of carp production, many fish species feed on both zooplankton and zoobenthos as adults, while larvae and fry rely mostly on zooplankton. It provides them with high quality nutrients and other molecules such as proteolitic enzymes, hormones and growth factors, which support digestive processes in immature larval gut.
Larviculture, especially start feeding of early larval stages, appear to be a critical point in fish culturing. However, artificial diets formulated for larvae show poor digestibility. Use of live feed of small dimensions, good nutritive characteristics and locomotion that provides visual stimulus, overcome that problem. Zooplankters commonly used for mass production as live feed for fish larvae are Rotifers (*Brachionus plicatilis* and *B. calyciflorus*) and brine shrimp (*Artemia salina*).
Shortly after hatching, carp larvae are transferred to nursing ponds for further rearing. Ponds are previously prepared through agrotechnical measures in order to provide nutrient enrichment and stimulate production. During the first days of exogenous feeding, larvae consume only the smallest zooplankters such as Rotifers and naupliar larvae of Copepods, while later they move on to bigger pray as Cladocerans.
However, due to variable environmental conditions, predation, competition or infections in earthen ponds, mortality in nursing ponds is often very high, reaching over 90%. Thereby, modern approach suggests rearing in indoor tanks in controlled systems and use of automatic feeding systems to provide precise portions of zooplankton.
Key words*: zooplankton, larviculture, semi-intensive system*

INTRODUCTION

Zooplankton is an important component of nutrition and protein source for both larvae and adults of many aquaculture species cultured in all three main types of production systems: extensive, semi-intensive and intensive. As live feed, they have crucial role in nutrition of early life stages of many fish species particularly for first exogenous feeding, but also play an important role as natural food for juveniles, adults and marketable size fish.
Main groups of zooplankters that may be found in earthen carp ponds are Protozoans, Rotifers and two groups of Crustaceans: Copepods and Cladocerans. In older ponds, they develop partly from egg bank stored in top layers of the sediment, and partly arrive through water bodies used for water supply (Dulic et al. 2011). Zooplankton dynamics depends on primary production of phytoplankton, that is determined by nutrient content in pond waterbody, in particular phosphorous and nitrogen. Nutrients derive from various sources – surface and underground waters, pond sediment, biochemical processes in water bodies, but also agrotechnical measures applied in earthen carp ponds. (Vollenweider 1968, Biro 1995). Larger zooplankters, cladocerans and copepods, show pronounced seasonality in abundance throughout the year, with maximum development during spring and early summer. In midsummer, when environmental conditions in fish ponds are optimal for carp growth, there is usually a decline in zooplankton abundance that develops strong need for added feed in order to maintain maximal growth of cultured fish (Dulic 2007, Dulic et al. 2009).
Zooplankton represents a high-quality food for fish larvae regarding its nutritive content. It provides them with high quality proteins, amino acids, lipids, fatty acids, vitamins, minerals and enzymes (Kibria et al. 1997) but also proteolitic enzymes, hormones or their regulators

and some nutritional growth factors which support and enhance digestive processes in immature larval gut. Biochemical composition and therefore nutritive quality of zooplanktonic organisms are genetically determined, but also influenced by their origin, life stage, season and type of ingested food (Mitra et al. 2007).
In aquaculture production, start feeding of early larval stages appears to be the most critical point. Some fish species, such as salmonids, have structurally and functionally fully developed digestive system before switching from endogenous to exogenous feeding, which enables them to digest complex molecules in artificial feed (Dabrowski 1984, Lavens and Sergeoloos 1996). Those species pose less of a problem with initial feeding, but many other species, common carp being one of them, have immature alimentary tract at larval stage. They are able to digest only very simple food particles at first feeding and require live planktonic organisms as first food (Garcia-Ortega et al. 1998). Although a lot of effort has been put in formulating artificial feed for larval stages and encouraging results have been obtained, live feed is still accepted as most suitable feed for most aquaculture species, which provides better growth and survival of early larval stages (Mitra et al. 2007, Sales 2011).
During last two decades, many reaserch have been done on developing alternative feed, mostly as microparticulate diets (MPD), but more investigations are needed to develop the right formulation which will provide results as good as live feed (Callan et al. 2003, Lazo et al. 2000).

LARVICULTURE AND PRODUCTION OF LIVE FEED

During first few days of early life, carp larvae feed on egg sac content. After absorption of yolk, before total depletion of egg sac, they start feeding exogenously. During first ted days larvae consume small zooplankters such as Rotifers and naupliar larvae of Copepods. After that time, they shift to consume bigger pray, as Cladocerans or zoobenthic organisms. Upon one month of age, carp juveniles show strong dietary preference for Cladocerans (Billard 1999), which also dominate in nutrition of older juveniles and adults, along with benthic fauna (mainly Chironomids and Oligochaeta), aquatic plants and some phytoplankton groups (Sakia and Das 2009).
In semi-intensive production systems, carp larvae are usually transferred to outdoor nursing ponds shortly after hatching. The size of the ponds may vary, depending on the time of rearing (usually from 3 to 6 weeks), prior transferring the fry into growing ponds for further culturing to marketable size. In order to provide optimal rearing conditions, ponds are previously prepared through application of different agrotechnical measures. Drying of the ponds, sunlight exposure and disinfection eliminate pathogens and prevent disease outburst, while fertilization with organic manures provide nutrient enrichment and stimulate production. (Markovic 2010). By use of insecticides, larger Copepods and Cladocerans may be removed from the ponds, thereby stimulating growth of small zooplankters preferred during first feeding of carp larvae.
Under natural conditions, effects of external factors plays dominant role in larval culturing. Due to variable environmental conditions, predation by frogs, birds or large insects, competition or infections in earthen nursing ponds, mortality often reaches as high as 90%. Therefore, intensive production system under controlled conditions is considered to be much more efficient in larviculture of carp. Indoor rearing in tanks, based on cultured zooplankters as live feed, provides reduction of losses, better growth and survival rate (Yoav et al. 2007). In closed systems, when external culture conditions are maintained within optimal range, larval performance at the initial feeding is mainly affected by husbandry practices - food, digestion and rearing density (Sharma and Chakrabarti 1999). Beside enhanced fingerling production, indoor rearing allows off-season seed production for better use of growing season and economical efficiency. During past decades, following enhancement of intensive production methods, using cultured live food, larvae production has rapidly increased (Barr et al. 2007).
Production and culturing of live feed is reported to be relatively easy (Lavens and Sorgeloos 1996, Moretti et al. 1999). The only zooplankters commonly produced in mass quantities are

two species of rotifers (*Brachionus plicatilis* and *B. calyciflorus*) and brine shrimp (*Artemia salina*). Rotifers are widely used as first feed for marine fish larvae and may be cultured and harvested in automated systems at high densities (Hagiwara et al. 2001). Freshly hatched *Artemia* nauplii are generally used as alternative to natural zooplankton in intensive carp larviculture, but costs of their production may be very high, reaching up to 79% of total expenses in larviculture (Kolkovski 2001, Stankovic et al. 2011). Rising costs of *Artemia* cysts of high hatching quality has put more focus on developing alternatives, as use of decapsulated cysts as direct food or pond grown zooplankton for live feed in hatcheries in different regions (Vanhaecke et al. 1990, Evjemo et al. 2003). When maintained on organic manures in ponds or tanks, production costs for live feed may be significantly decreased. Wastewaters or possibly effluents from fish ponds may also be used for zooplankton culture, providing low-cost production system and decreasing environmental pollution from aquaculture (Kibria et al. 1999).

LARVAL NUTRITION AND FIRST FEEDING

Processes of digestion, absorption and assimilation as well as nutritional requirements of fish differ significantly among life stages. After hatching, digestive system of carp larvae is still immature and developing, represented by histologically undifferentiated straight tube, supplied with few active enzymes able to digest only very simple food particles (Dabrowski 1984). Each enzyme develop independently during ontogenesis, and show relatively lower activity at early stages compared to adults. In such species, when digestive system is not fully functional at first feeding, transition to external food is more difficult and usually require live feed as part of their diet (NRC 1993). Poor digestion of artificial diets with complex molecules of proteins, carbohydrates and other nutritional components, shown at this life stage, is partly caused by low affinity of proteolitic enzymes for the proteins present in formulated diets, but also absence of some enzymes, hormones or their regulators, and growth factors that are naturally present in live feed (Dabrowski 1984, Lauff and Hofer 1984). Proteolitic activity of the carp larvae depends on the developmental stage and nature of food (Sharma and Chakrabarti 1999). Through consumption of zooplankton, carp larvae are not only supplied with their enzymes (proteinases, peptidases, amylases, lipases) and other molecules that enhance digestion processes, but also activation of zymogens present in undeveloped digestive tract takes place. Protein and lipid fractions extracted from live feed may serve as precursors or activators in assimilation processes. Furthermore, high water content makes zooplankters easily digestible, unlike artificial feed with 60 to 90 percent of dry matter (Kolkovski 2001).

The size of the mouth gape determines dimensions of feed particle that fish larvae may consume. This variable is correlated to body size, egg diameter and duration of endogenous feeding. Carp larvae have small yolk sac which is usually absorbed in 3 to 5 days after hatching. At first exogenous feeding they can only consume particles lower than 0,5 mm (Dabrowski and Bardega 1984), weighing daily 50 to 300 percent of their body weight, compared to 2 to 10 percent of body weight for adults (NRC 1993). Small dimensions of cultured zooplankters (50 to 200 µm for *Brachionus* sp. and 400 to 550 µm for *Artemia salina*), their nutritive characteristics, digestibility and constant locomotion, that provides a visual stimulus, puts live feed ahead of inert compound diets for first feeding of carp larvae (Rønnestad et al. 1999, Kolkovski 2001).

Different sources of zooplankton, according to culture conditions and their geographical origin, may have variable nutritional quality, mostly related to fatty acid composition (Dabrowski 1984, Watanabe et al. 1983). In freshwater zooplankton, lipid content is influenced by seasonal dynamics and succession of phytoplankton species and may significantly differ between populations (Jeffries 1970). Fatty acid content and amount present in live food strongly affect growth and survival of fish larvae, which are very sensitive to deficiency of (n-3) PUFA, polyunsaturated fatty acid (Watanabe et al. 1983, NRC 1993). Therefore, enrichment of zooplankton organisms with fatty acids through special nutrition or by using lipid suspensions, in some cases may be necessary to compensate for differences

between natural and cultured live feeds, in order to provide quality production of larvae and juveniles (NRC 1993, Yoav et al. 2007).

CONCLUSION

A lot of research has been done on formulating artificial feed for first feeding of fish larvae. These attempts consider not only the right combination of ingredients and their digestibility, but also ontogenetic development of digestive system and enzyme activation (Kolkovski 2001). However, dietary needs of growing larvae differ significantly from those of adult fish. Use of artificial feed in early life stages often leads to stunted growth and high mortality, while use of live feed still provides much better performance of larvae of many aquaculture species, including common carp. Both frozen and freeze-dried zooplankton represent an excellent food source for hatchlings of many species which normally require live plankton for growth. Their nutritional quality fulfill the needs of young larvae and contribute to digestion and absorption processes resulting in better growth and survival rate then when fed on artificial feed alone.

ACKNOWLEDGMENTS

The present study was supported by the Ministry of Education and Science, through the project Improvement of production capacities of carp (*Cyprinus carpio)* through nutrition and selective breeding programs (TR 31075).

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EFFECT OF THE DIETARY CAMELINA MEAL ON LAYER PERFORMANCE

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ABSTRACT: A 60 days test on 90 Lohmann Brown layers aged 40 weeks was conducted to evaluate the effect of the dietary Camelina meal on layer performance. The hens was housed in cages (3 layers/cage) and assigned to three groups (C, E1 and E2). Feeding was ad libitum with one meal per day. The control diet (C) had as basal structure: corn (57.47%), soybean meal (15%), corn gluten (4 %) and sunflower meal (9.5%). The experimental diets (E1, E2) were differentiated from C by inclusion of Camelina meal in percentages of 3% (E1) and 6% (E2) respectively. Throughout the experimental period the feed intake, egg production, egg weight and egg quality parameters were recorded on a daily basis. The results show that the feed conversion ratio (g feed/g egg) of group C (1.908±0.10) was significantly (P≤ 0.05) higher than in E1 (1.883±0.10) and E2 (1.817±0.15). However, egg production of E2 (93.68±2.26%) was significantly (P≤ 0.05) lower than in the other 2 groups (97.22±1.76% for C; 97.99±1.99 for E1). The weight of E2 eggs (64.00±2.31 g/egg) was comparable with the weight of C eggs (64.61±2.48).
Key words: *layers, Camelina, meal, intakes, egg*

INTRODUCTION

Camelina sativa is an oil seed crops in the Brassica family (Cruciferae) with mustard, cabbage, rapeseed, broccoli, turnips, Brussels sprouts, cauliflower that has an oil content of approximately 40% (Cherian and col. 2009). Is known as "false flax" or "gold of pleasure". Originally from Northern Europe and Central Asia. Camelina (Camelina sativa) is considered a low-input culture requiring small amounts of water and reduced fertility compared to other species of nuts (Putnam et al., 1993). Can be used as a rotation crop for wheat. The plant has gained popularity in recent years due to the discovery that camelina oil can be used as biofuel for aircraft industry.

Camelina meal as an animal feed additive has been studied and was found beneficial in increasing the ω-3 fatty acid content in eggs and the meat quality of laying hens and broiler chickens (Ryhanen et al., 2007, Rokka et al., 2002). Camelina meal is a new byproduct that remains after oil extraction for biodiesel production and might be considered as an alternative to flaxseed for poultry nutrition as an n-3 fatty acid source because of the residual oil (Zubr, 1997). If growing camelina becomes a viable crop, the integration of the resultant meal into local diets would further increase the value of the crop. Camelina meal typically contains 10-15% residual oil and 40% crude protein, minerals and 10-12 % crude fiber and has a moderately-low glucosinolate content.

Previous studies (Cherian et al., 2009) on the nutrient composition of camelina meal (a byproduct obtained from camelina seeds after oil extraction) indicated that the meal contains between 35-40% PB, 4688 kcal/kg EB, 11 to 12% fat, α-linolenic acid (n-3) representing 30% of total fatty acids. Omega-3 polyunsaturated fatty acids play an important role in brain function, in growth and body development. It also reduces the risk of heart disease, reduce inflammation and may improve chronic disease risks (heart disease, cancer and arthritis. Some of the symptoms of deficiency of omega-3 are: poor memory, dry skin, heart problems, mood swings and depression, circulatory problems.

Regarding the use of camelina as a forage crop, due to its high content in linolenic acid (20-39%) and being considered a low-input ingredient, camelina began to be regarded as a staple fodder. For inclusion in compound feed as forage plant, seeds, oil, and especially camelina meal can be used. This happened after the Directive 2008/76/EC of 25 July 2008 when camelina was removed from the list of undesirable substances in feed intended for animals kept in intensive system rearing, due to the EFSA opinion (European Food Safety Authority) of November 27, 2007.
In other studies by Cherian and col., (2009), and Aziza al., (2010), Camelina meal was used 10% in laying hens and broilers ratio. These authors reported a significant increase in omega-3 fatty acids in eggs and in the muscle tissues of broiler chickens. In addition to omega-3 polyunsaturated fatty acids, the camelina meal contains other bioactive compounds such as tocopherols and phenolic (Matthäus, 2002, Salminen et al, 2006). This phenolic compounds can help prevent lipid oxidation both in combined fodder and in eggs or meat obtained from birds fed rations high in polyunsaturated fatty acids (McCarthy et al, 2001; Cherian et al. 2002).
Researchers Amy Batal and Nick Dale (2009) have shown that completely new ingredients became available for feed manufacturing industry, so in 2009, has been shown a particular interest concerning camelina meal utilisation. Based on the analyzes performed in laboratories at the University of Georgia, Athènes (USA) on the camelina meal, Batal and Dale (2009) included this variety in *Ingredient Analysis Table: 2010 Edition*.
The aim of the paper is to assess the effect of 3 and 6% Camelina meal addition in laying hens diets, on their bioproductive parameters.

MATERIAL AND METHODS

The experiment was conducted on a total of 90 laying hens, light breed, aged 40 weeks, Lohmann Brown hybrid for a period of 60 days. The animals were divided into 3 groups: control (C) and two experimental (E1, E2), housed in battery structured on three levels allowing to register the daily intake and daily egg production. Experimental hall lighting was provided by a scheme with up to 16 hours daily light and temperature was maintained at 25° C throughout the experimental period. For this experiment, were produced 3 types of feeds: a control variant (CF 21-5) and 2 experimental variants, E1 (with an inclusion rate of 3% of camelina meal) and E2 (with an inclusion rate of 6% of camelina meal). All these types of feeds and raw materials that went into their component analyzes were subjected to determine their chemical composition. Thus, we determined DM, CP, Fat, Fiber, ASH, amino acids, fatty acids according to the ISO methods (The Romanian Standardized Association-ASRO- Standardized Bulletin, 2010): SR ISO 6496/2001; ISO 5983-2/2009; ISO 6492/2001; ISO 6865/2002; ISO 2171/2010; SR ISO 13903/2005; SR ISO/TS 17764-2/2008. Also it were determinated the degradation of fat (index peroxide ml thiosulphate 0.01N / g fat, acidity fat mg KOH / g fat and KREISS reaction) according to STAS 12266-84.
Feeding was done ad libitum in a single allowance / day. Daily amount of feed given was weighed separately for each cage and the fodder left uneaten was also weighted (in the morning). Consumption were recorded daily, health status and egg production. Eggs were weighed daily. Chickens were weighted at the beginning and at the end of the experiment.
During the experiment were collected 4 sets of eggs / batch of 18 samples each, from each lot, for a period of 5 days, every 2 weeks. A number of 3 eggs were used of the 18 samples collected in order to achieve an average sample, resulting in 6 samples / group, from which separate samples of white, yolk and shell were taken. At all harvesting, egg physical parameters were determined: egg weight, yolk weight, weight whites, shell weight, color intensity, degree of freshness, pH yolk and whites and shell thickness, using standard methods for determination. For determination of statistical data of the experimental results regarding zootechnical and eggs quality parameters it has been used STATVIEW soft. All these determinations were made in the Laboratory of Chemistry and Physiology of Nutrition - IBNA Balotesti.

RESULTS AND DISCUSSIONS

To establish the nutrient value of camelina meal used in experimental laying hens feeding, chemical composition and gross energy have been determined alongside the fatty acids concentration (Table 1).

Table 1. Chemical composition and fatty acids concentration of camelina meal

Item	Camelina meal
DM, %	91.0
CP, %	36.47
Fat, %	7.48
Fiber, %	10.84
ASH, %	5.58
GE, Kcal/Kg	4555
Fatty acids, g % fat sample	
C14:0	0.10
C16:0	7.11
C16:1	0.26
C18:0	2.39
C18:1	20.88
C18:2n-6	24.49
C18:3n-3	23.65
C20:0	1.53
C20:1	11.38
C18:4n-3	0.92
C20:2n-6	1.39
C20:4n-6	0.66
C20:5n-3	0.41
C22:1n-9	3.21
C22:5n-3	0.21
Other fatty acids	0.32
Σ SFA	11.13
Σ MUFA	35.73
Σ PUFA	51.73
n-3:n-6	0.949

Where: Σ SFA – total saturated fatty acids;
Σ MUFA – total monounsaturated fatty acids;
Σ PUFA – total polyunsaturated fatty acids.

Camelina meal was found to be rich in linoleic (18:2n-6) and linolenic acid (18:3n-3), and these constituted 24.76 and 23.91%, respectively. Oleic acid (18:1) and eicosenoic acid (20:1) constituted 21.11 and 11.51%, respectively, in the Camelina meal. The n- 3 fatty acid and n-6 fatty acid content of Camelina meal makes it a potentially suitable plant of essential polyunsaturated fatty acid source in poultry diets (Aziza et al., 2010).
Amino acids composition (Table 2) of the camelina meal was determinated. Lysine, methionine, and cystine are usually the first-limiting acids in poultry nutrition, which makes Camelina meal a potential source of protein for poultry (Aziza et al., 2010).

Table 2. Amino acids composition of the camelina meal

Item	Camelina meal g/100 g camelina meal
Aspartic Acid	3.000
Glutamic Acid	6.289
Serine	1.531
Glycine	1.483
Threonine	1.520
Arginine	2.139
Alanine	1.659
Tyrosine	0.756
Valine	1.981
Phenylalanine	1.573
Isoleucine	1.452
Leucine	2.279
Lyzine	1.678
Cystine	0.739
Methionine	0.879
Total amino acids	**28.959**
CP, %	**36.47**

The structure of rations (Table 3) was calculated based on determinations of chemical composition of feed materials using a mathematical model of poultry feeding structure (Burlacu et al., 1999) and in accordance with nutritional requirements (NRC, 1994) recommended for this category of intensive poultry rearing.

Table 3. Rations structure fed to laying hens

Ingredient	C %	E1 %	E2 %
Corn	57,47	62,23	61,64
Sunflower meal	9,5	2,6	2,7
Soybean meal	15	16,2	13,7
Corn gluten	4	4	4
Vegetable oil	2	0	0
Camelina meal	0	3	6
CaCO3	9,05	9,05	9,05
Monocalcium phosphate	1,4	1,38	1,36
Salt	0,3	0,3	0,3
Methionine	0,13	0,12	0,1
Lisine	0,1	0,07	0,1
Choline	0.05	0.05	0.05
Commercial premix	1	1	1
Total	100,00	100,00	100,00
Analized			
GE (kcal/kg)	3886	3894	3881
DM	89.83	89.71	89.58
CP	20.28	19.38	20.02

Fatty acids concentration is presented in Table 4. Fatty acids C18: 3n-3 is significantly higher in groups E1 and E2 while fatty acids C18: 2n-6 did not differ within the composition of these 3 types of feeds. The high concentration of linolenic acid, especially in combined fodder of E2 variant makes that ratio between fatty acids n-3: n-6 to be significantly better in this group compare to C (0.05) or E1 (0.08). Total PUFA is higher in the experimental rations compared with a control group. In addition to linolenic acid, there are other fatty acids that differentiates, respectively, C18: 4n-3 and C22: 5n-3 all in favor of the experimental variants.

Table 4. Fatty acid concentration of the experimental diets

Fatty Acids	C g % fat sample	E1 g % fat sample	E2 g % fat sample
C18:2n6	44.60	44.37	43.03
C18:3n3	1.71	2.87	4.34
C18:4n3	0.06	0.17	0.26
C20:5n3	0.37	0.33	0.32
C22:5n3	0.12	0.18	0.18
Other fatty acids	53.13	52.08	51.87
Σ SFA	16.49	19.45	18.7
Σ MUFA	35.89	32.02	32.3
Σ PUFA	46.86	48.05	48.5
n-3:n-6	0.05	0.08	0.12

Where: Σ SFA – total saturated fatty acids;
Σ MUFA – total monounsaturated fatty acids;
Σ PUFA – total polyunsaturated fatty acids.

Evidence of fat degradation, meaning index peroxide ml thiosulphate 0.01N / g fat (admitted limit 1.2), acidity fat mg KOH / g fat (admitted limit 50) and KREISS reaction (admitted limit - negative) was calculated (Table 5). Recorded value at E2 group (the richest in PUFA, Table 4) for fat acidity was above the allowable. The other parameters of degradation of fat were normal.

Table 5. Fat indices degradation

Index	C	E1	E2
Index peroxide ml thiosulphate 0.01N / g fat	0.58	0.95	0.89
Acidity fat mg KOH / g fat	36.28	48.09	54.71
KREISS reaction	negative	negative	negative

Introduction of camelina meal in poultry diets (3% and 6% in experimental groups), impacted zootechnical parameters (Table 6).

Table 6. Zootechnical parameters obtained, averages / group / experimental period

Item	C	E1	E2
Average Daily Intake *, g/day/hen	123.26±7.52	117.20±6.58	116.27±12.46
Egg production **, %	97.22±1.76	97.99±1.99	93.68±2.26
Egg weight *, g	64.61±2.48	62.36±1.81	64.00±2.31
Feed conversion **, g feed/g egg	1.908±0.10	1.883±0.10	1.817±0.15

**no significant differences among treatment means; **significant differences among treatment means*

Note that the registered laying percentage of the experimental group E2 (6% camelina meal) was significantly ($P \leq 0.05$) lower compared with that obtained from other 2 groups (C, E1 respectively). When Camelina meal constituted more than 10% of the diet fed to laying hens, a reduction in egg production was observed (Cherian, 2009). Also, the feed conversion ratio (g feed/g egg) of group C (1.908±0.10) was significantly ($P \leq 0.05$) higher than in E1 (1.883±0.10) and E2 (1.817±0.15). For the other 2 parameters (average daily intake and egg weight) no significant differences were registered. Decreased feed intake during the starter phase in birds fed Camelina cake or Camelina meal were reported by Ryhänen et al., 2007 and Pekel et al., 2009. The weight of E2 eggs was comparable with the weight of C eggs (Table 6).
The results of weight components and physical parameters of the eggs collected during the experiment are presented in Table 7.

Table 7. Data on weight components and physical parameters determined on eggs (valori medii/lot)

Item	C	E1	E2
Albumen weight, g	38.97±2.82	37.46±3.35	38.46±2.79
Yolk weight, g	17.14±0.34	16.88±0.21	17.17±0.25
Eggshell weight, g	8.50±0.54	8.02±0.42	8.37±0.34
Thickness eggshell, mm	0.34±0.02	0.34±0.02	0.34±0.02
pH albumen	8.75±0.20	8.84±0.12	8.87±0.18
pH yolk	6.14±0.06	6.16±0.07	6.10±0.02
Yolk colour intensity*	5.61±0.96	6.00±0.92	6.40±0.88

*The yolk color is defined according to the Roche Yolk color Fan and colors should be withing the range 1 to 15 (1 represent bright yellow color and 15 represent dark yellow color)

No difference due to diet was observed for weight components and physical parameters determined on eggs.

CONCLUSION

The compound feed incorporated with camelina meal was found to be rich in PUFA and could be used into local poultry diets as a source of energy, protein and especially essential fatty acids.
Introduction of camelina meal in poultry diets (3%) has not negatively impacted zootechnical parameters but a higher percentage (6%) affected significant the egg production.

ACKNOWLEDGEMENTS

This paper is a result of international cooperation, FP7 REGPOT-2007-3 project Feed-To-Food GA No 207043 funded by EC and the research within the project III46012 financed by the Ministry of Education and Science, Republic of Serbia.

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EFFECT OF DIETARY TREATMENTS ON THE FATTY ACID COMPOSITION AND SENSORY CHARACTERISTICS OF CHICKEN MEAT

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ABSTRACT: The research was carried out on in two repetitions on Ross 308 male hybrid chickens. Chickens were divided into five groups (five dietary treatments differing in source and concentration of plant oil and fish oil). Feeding chickens with diets of determined composition from 22nd to 42nd day of fattening resulted in altered fatty acid profile in white meat and dark meat, and in significantly lowered n-6/n-3 PUFA (polyunsaturated fatty acids) ratio in white meat. The respective ratio was lowered from 10.85 to 2.67 in white meat, and from 12.14 to 3.27 in dark meat. Trained sensory panel was assessing the samples using scale from 0 (imperceptible) to 9 (extremely perceptible), according to the intensity of the following sample characteristics: chicken odour and flavour, fishy odour and flavour, and rancid odour and flavour. Overall impression was assessed by scale from 1 (poor) to 5 (typical). Characteristics of chicken odour and flavour were graded as of moderately high intensity in dark meat and of white meat, and there was no difference determined between the control and experimental treatments. Slightly noticeable rancid odour and flavour was determined in all meat samples, however the differences were not statistically significant ($P>0.05$). Although all experimental treatments were assessed as having more fishy odour and flavour than the control sample, statistically significant difference between samples was found only for fishy flavour in dark meat ($P<0.05$). In repetitions, upon altering the feeding treatment (without fish oil), and seven days prior to slaughtering of chickens, the intensity of fishy odour and flavour was assessed with "none" to "slightly noticeable", and differences between samples were not statistically significant ($P>0.05$). This research proved that, if applying certain dietary treatments, it was possible to produce chicken meat of satisfactory sensory characteristics enriched with n-3 PUFA, with n-6/n-3 PUFA ratio of 4.48 in white meat and of 4.57 in dark meat.
Key words: *chicken, meat, FA profile, n -3 PUFA, sensory characteristics*

INTRODUCTION

Anthropological and epidemiological studies indicate that humans have evolved with respect to nutrition which had n-6 PUFA / n-3 PUFA ratio of 1, while today that ratio in so called western nutrition amounts from 15/1 to 16.7/1 (Calvani and Benati, 2003; Simopoulos, 2006). Similar values (17.1/1) were also obtained in some studies in Croatia (Kralik et al., 2001; Primorac et al., 2003). High PUFA n-6/PUFA n-3 ratio is related to occurrence of many diseases - cardiovascular, inflammatory and autoimmune diseases, and cancer (Connor, 2000; Simopoulos, 1998, 2006; Mozaffarian, 2008). This is the reason why animal products, which are mostly consumed and which fatty acid content can be easily modified, tend to be subject to increase of n-3 PUFA portion, especially of long-chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Lopez-Ferrer, 1999). However, increased portion of these acids in animal feed, and consequently in a product, can cause unwanted changes in sensory characteristics, which is also affecting product acceptability (Scaife et al., 1994; Lopez-Ferrer, 2001) and effects the meat quality (Wood et al., 2003). The aim of this study was to determine in what way different combinations and concentrations of plant oils and oil preparations enriched with EPA and DHA affected fatty acid profile in meat and its sensory characteristics.

MATERIAL AND METHODS

The research into effects of dietary treatments on sensory characteristics of meat was carried out on Ross 308 chickens within two experiments. Dietary treatments in the 1st experiment:

- K (control) 5% sunflower oil;
- A 2.75% sunflower oil, 2% linseed oil, 0.25% PBE1 oil;
- B 2.75% sunflower oil, 2% linseed oil, 0.25% PBE2 oil;
- C 2.50% sunflower oil, 2% linseed oil, 0.5% PBE1 oil;
- D 2.50% sunflower oil, 2% linseed oil and 0.5% PBE2 oil.

Dietary treatments in the 2nd experiment:

- K (control) 3% soybean oil;
- A 3% linseed oil;
- B 2.9% linseed oil and 0.1% PBE1 oil;
- C 2.9% linseed oil, 0.1% PBE2 oil.

Seven days prior to slaughtering chickens were fed diets without PBE1 and PBE2 oil. PBE1 is oil of sea organisms containing 15.36% EPA and 9.99% DHA, and PBE2 is also oil of sea organisms with 10.55% EPA and 22.10% DHA.
Chickens were slaughtered after 42 days of fattening. Samples of white meat (breasts) and dark meat (drumsticks with thighs) were taken for chemical analysis. Fat content was determined by the Stoldt method (Hungarian Standard No. 6830-66). Fatty acid profile in meat samples was determined by the Chrompack CP 9000 chromatograph equipped with flame ionization detector. From the homogenised sample, dependent on fatty acid composition, 2-4 g was measured for fatty acid composition in a glass equipment and 5 cm^3 n-heptane and in order to eliminate water content of the sample 2-4 g dry sodium-sulphate was added to it. From the heptane solution containing fat that is free from water, 0.5 cm^3 was pipetted to a test tube, and 0.5 cm^3 sodium-metilate was added to it, the mixture was heated at 60 ^oC for 60 minutes, and during this time the mixture was shaken up intensively every 10 minutes. After this 1 cm^3 n-heptane and 1 cm^3 distilled water was added to the mixture, it was homogenised, the heptane phase was separated from the water phase, (the excess of the reagent was removed), the heptane phase was dried by dry sodium-sulphate, and dependent on fat content of the sample, 0.5-2.0 μl was injected into the column of the gaschromatograph (300 x 025 mm, quartz capillary). The humid phase (active phase) was Cp-Sil-88. The temperature of the injector and the flame ionisation detector was 220^oC, the temperature of the column at the start was 100^oC, the speed of the warm up of the column was 6^oC /minute until 210^oC, and this temperature was used at the end of the analysis. For quantitative evaluation, the weight percentage proportions of the methyl esters were regarded as equal to the proportions of the corresponding peaks in the chromatogram. The fatty acid composition for unknown samples was calculated as a function of the comparative mass percentage of fatty acid methyl-esters (Csapó et al., 1986). Cooled, boneless and frozen meat was stored for 30 days at -20^oC until sensory analysis. Thawing was carried out in refrigerator for 16 hours. Preparation / roasting and sensory analysis of white and dark meat were done separately. Meat samples of each treatment were separately wrapped in aluminum foil and roasted in the oven at a temperature of 175^oC, 60 minutes for white meat and 50 minutes for dark meat. Based on previous research, these conditions were proved as optimal. Before serving, the samples were cut into pieces and heated in glass containers with lids in a water bath at temperature of 55-60^oC. Samples (20 g) were served in covered plastic containers labeled with three-digit number. Panel of seven selected assessors evaluated the intensity of following sample characteristics: chicken odour and flavour, fishy odour and flavour and rancid odour and flavour, using scale from 0 (imperceptible) to 9 (extremely perceptible), while the overall impression was assessed by scale from 1 (poor) to 5 (typical). All samples were served simultaneously. Assessment was carried out in 4 replications with different coding, from 2 to 4 pm. During training that lasted for several days panelists were

presented samples of chicken meat from different dietary treatments, and different types of oil (fish, linseed, sunflower and rancid oils), for the purpose of developing common terminology and scale usage (Meilgaard et al., 2000Average values and standard deviations were calculated for each parameter ($\bar{x} \pm$SD). Sensory analysis data were tested by Friedman's test, while the fatty acids content was tested by ANOVA, both using Statistica 7.0 software.

RESULTS AND DISCUSSION

Analysis of fatty acid profile in chicken meat lipids (Table 1) from the 1st experiment showed that both meat types changed as a consequence of n-3 PUFA and n-6 PUFA ratio in dietary treatments. White and dark meat of the control (K) contained 3.35% and 3.54% of n-3 PUFA, respectively, while portions of EPA (0.09% and 0.12%, respectively) and DHA (0.91% and 0.65%, respectively) were insignificant. White meat enriched with n-3 PUFA contained 0.62% - 0.85% EPA (P=0.005) and 1.74% - 4.62% DHA (P=0.002) in total content of fatty acids. Dark meat also exhibited identical changes in the fatty acid profile. It contained 0.49% - 0.80% EPA and 1.34% - 2.61% DHA (P=0.01) in total content of fatty acids, as depending on the dietary treatment. White and dark meat of the control contained only 0.09% and 0.12% EPA, respectively, and 0.91% and 0.65% DHA, respectively. The n-6/n-3 PUFA ratio in white meat of the control was 10.85, and in dark meat it was 12.14. Application of described dietary treatments the mentioned ratio was lowered from 2.67 - 3.05 in white meat, and from 3.27 – 3.50 in dark meat.

Table 1. Fatty acids content in chicken meat (1st experiment)

fatty acids, %	**dietary treatments – groups ($\bar{x} \pm$SD)***				
	K	**A**	**B**	**C**	**D**
white meat					
Σ SFA (P=0.11)	34.49±4.66	30.53±3.68	30.25±5.04	32.26±3.51	27.07±3.46
Σ MUFA (P=0.09)	19.12±4.04	22.34±1.90	19.06±2.55	19.57±2.20	19.67±2.53
Σ n-6 PUFA **(P=0.02)**	36.24±3.72	32.42±1.97	33.73±4.33	32.92±1.80	37.47±2.28
Σ n-3 PUFA **(P=0.001)**	3.35±0.25	11.34±0.63	11.08±1.56	11.08±0.79	14.08±0.80
αLNA (P=0.52)	1.49±0.48	6.67±1.43	5.59±1.50	5.60±1.45	6.58±2.11
EPA **(P=0.005)**	0.09±0.03	0.63±0.10	0.62±0.13	0.67±0.28	0.85±0.13
DHA **(P=0.002)**	0.91±0.26	1.74±0.61	2.86±0.88	2.63±0.63	4.62±1.61
Σn-6 P/ Σn-3 **(P=0.005)**	10.85±1.25	2.86±0.15	3.05±0.13	2.99±0.12	2.67±0.25
dark meat					
Σ SFA (P=0.89)	27.26±1.59	25.80±1.96	26.33±2.07	25.47±1.77	26.65±4.78
Σ MUFA (P=0.06)	22.91±1.62	23.49±2.36	20.92±1.38	21.38±0.98	21.13±1.88
Σ n-6 PUFA (P=0.41)	42.92±2.06	37.69±1.16	39.59±1.56	39.21±1.54	37.94±3.84
Σ n-3 PUFA (P=0.54)	3.54±0.18	11.47±0.45	11.34±0.56	11.81±0.63	11.58±0.61
αLNA (P=0.39)	2.18±0.25	7.93±1.22	6.65±1.33	7.37±0.75	7.21±1.60
EPA **(P=0.01)**	0.12±0.02	0.49±0.08	0.56±0.09	0.80±0.09	0.59±0.06
DHA **(P=0.01)**	0.65±0.13	1.34±0.46	2.49±0.70	2.12±0.41	2.61±0.76
Σn-6 / Σ n-3 (P=0.14)	12.14±0.55	3.29±0.08	3.50±0.23	3.33±0.17	3.27±0.21

*mean and standard deviation of 10 replications

Sensory assessment of meat samples (Table 2) showed that differences between samples for the characteristic of chicken odour and flavour and rancid odour and flavour were not statistically significant (P>0.05) for either white meat or dark meat.

All dark meat samples were assessed as having more fishy odour and flavour than the control, but statistically significant difference was only for flavour (P<0.05).

White meat samples were also noticed to have more intensive fishy odour and flavour than the control samples, however, the differences were not statistically significant (P>0.05).

This could be explained by the fact that chicken dark meat contains more fat than white meat, so the characteristic of fishy flavour was more expressed in this type of meat.

According to overall sensory assessment, control sample was better assessed than other samples of both white and dark meat, although differences were not statistically significant (P>0.05).

Table 2. Effect of dietary treatments on the sensory characteristics of chicken meat (1st experiment)

characteristic	dietary treatments – groups ($\bar{x}$ ± SD)*				
	K	A	B	C	D
white meat					
chicken odour (P=0.69)	5.9 ± 2.1	5.5 ± 2.0	5.8 ± 1.6	5.5 ± 2.3	5.3 ± 2.0
fishy odour (P=0.30)	0.5 ± 0.8	1.5 ± 0.6	0.8 ± 0.7	1.7 ± 0.9	1.5 ± 1.0
rancid odour (P=0.35)	0.8 ± 0.7	0.7 ± 0.6	0.8 ± 0 7	0.4 ± 0.4	0.4 ± 0.5
chicken flavour (P=0.72)	5.7 ± 2.2	5.2 ± 2.3	5.5 ± 2.1	5.0 ± 2.5	5.1 ± 2.3
fishy flavour (P=0.09)	0.2 ± 0.3	0.6 ± 0.7	0.5 ± 0.6	1.4 ± 1.4	1.0 ± 1.0
rancid flavour (P=0.77)	0.6 ± 0.7	1.0 ± 1.1	0.7 ± 0.8	0.7 ± 0.8	0.8 ± 0.9
overall impression (P=0.61)	3.8 ± 0.8	3.1 ± 0.9	3.5 ± 0.7	3.0 ± 1.2	3.0 ± 1.0
dark meat					
chicken odour (P=0.34)	6.1 ± 2.2	5.0 ± 2.6	5.4 ± 1.8	5.5 ± 2.4	5.8 ± 1.9
fishy odour (P=0.61)	0.8 ± 0.9	1.7 ± 1.1	1.4 ± 0.7	1.5 ± 1.1	1.3 ± 0.7
rancid odour (P=0.93)	1.0 ± 1.0	0.8 ± 0.4	1.1 ± 0.7	1.0 ± 0.9	1.0 ± 0.6
chicken flavour (P=0.22)	6.2 ± 2.3	5.0 ± 2.4	5.5 ± 1.9	5.4 ± 2.1	5.4 ± 1.7
fishy flavour (P=0,04)	**0.3 ± 0.6**	**1.8 ± 1.2**	**1.2 ± 0.8**	**1.4 ± 0.9**	**1.3 ± 0.8**
rancid flavour (P=0.46)	1.2 ± 0.8	0.8 ± 0.5	0.8 ± 0.7	1.0 ± 0.6	0.8 ± 0.8
overall impression (P=0.31)	3.8 ± 0.7	2.9 ± 1.0	3.1 ± 0.8	3.0 ± 1.0	3.1 ± 0.8

*mean and standard deviation of 4 replications

Analysis of fatty acid profile in chicken meat of the 2nd experiment (Table 3) proved that applied dietary treatments had effect on the deposition of some fatty acids in chicken muscle tissue. Increase of n-3 PUFA in chicken diets (treatments A, B and C) resulted in increase of those fatty acids in white and dark meat. The increase was mostly related to content of αLNA, as during the last 7 days of fattening chickens had diets with reduced content of EPA and DHA due to exclusion of PBE1 and PBE2 oils. That procedure aimed at improvement of organoleptic characteristics of chicken meat in order to avoid unwanted occurrence of fishy flavour in dark meat, which was the case in the 1st experiment.

Table 3. Fatty acids content in chicken meat (2nd experiment)

fatty acids, %	dietary treatments – groups ($\bar{x}$ ± SD)*			
	K	A	B	C
white meat				
Σ SFA **(P=0.04)**	32.54±3.42	29.94±1.86	29.94±1.59	28.09±2.38
Σ MUFA **(P<0.001)**	18.11±1.28	21.46±1.34	22.40±1.38	24.03±2.04
Σ n-6 PUFA (P=0.12)	38.84±3.49	37.39±1.76	35.73±0.82	36.30±2.18
Σ n-3 PUFA **(P<0.001)**	6.21±0.27	7.65±0.45	7.67±0.22	8.14±0.84
αLNA **(P<0.001)**	2.85±0.58	4.69±0.22	5.11±0.29	5.80±0.79
EPA (P=0.34)	0.46±0.05	0.23±0.10	0.21±0.03	0.21±0.04
DHA **(P=0.04)**	1.62±0.30	1.51±0.24	1.25±0.12	1.14±0.17
Σ n-6 / Σ n-3 **(P<0.001)**	6.28±0.77	4.89±0.22	4.66±0.11	4.48±0.21
dark meat				
Σ SFA (P=0.31)	22.12±1.34	21.25±0.78	21.45±0.75	20.92±1.38
Σ MUFA **(P=0.005)**	24.55±0.78	27.10±1.97	26.17±1.61	28.35±1.88
Σ n-6 PUFA **(P<0.001)**	46.34±1.37	42.13±1.44	42.62±1.62	41.02±2.29
Σ n-3 PUFA **(P<0.001)**	6.30±0.56	8.27±0.43	8.96±0.24	8.99±0.43
αLNA **(P<0.001)**	5.22±0.24	7.28±0.37	7.96±0.35	8.02±0.65
EPA **(P=0.03)**	0.42±0.03	0.11±0.02	0.15±0.02	0.16±0.02
DHA (P=0.15)	0.36±0.03	0.50±0.12	0.44±0.15	0.43±0.05
Σ n-6 / Σ n-3 **(P<0.001)**	7.38±0.44	5.10±0.14	4.76±0.09	4.57±0.13

*mean and standard deviation of 10 replications

All white and dark meat samples were assessed having strong chicken odour and flavour (Table 4), and slightly noticeable rancid odour and flavour but the differences between samples were not statistically significant (P>0.05), neither for chicken or rancid odour and flavour. Intensity of fishy odour and flavour was assessed as none to slightly noticeable, and slightly higher values were obtained in dark meat. Differences between samples were not statistically significant (P>0.05).

Table 4. Effect of dietary treatments on the sensory characteristics of chicken meat (2nd experiment)

characteristic	dietary treatments – groups ($\bar{x}$ ± SD)*			
	K	A	B	C
white meat				
chicken odour (P=0.16)	7.2 ± 0.7	7.1 ± 0.7	6.9 ± 0.6	7.0 ± 0.7
fishy odour (P=0.14)	0.4 ± 0.4	0.5 ± 0.7	0.6 ± 0.6	0.4 ± 0.5
rancid odour (P=0.59)	0.7 ± 0.8	0.5 ± 0.6	0.6 ± 0.6	0.9 ± 0.8
chicken flavour (P=0.27)	7.2 ± 0.7	6.9 ± 0.7	6.8 ± 0.8	6.7 ± 0.7
fishy flavour (P=0.11)	0.2 ± 0.3	0.5 ± 0.6	0.6 ± 0.7	0.3 ± 0.4
rancid flavour (P=0.46)	0.5 ± 0.6	0.5 ± 0.6	0.6 ± 0.7	0.6 ± 0.7
overall impression (P=0.17)	4.0 ± 0.4	4.0 ± 0.6	3.7 ± 0.6	3.7 ± 0.5
dark meat				
chicken odour (P=0.09)	6.9 ± 1.0	6.9 ± 0.5	7.4 ± 0.6	6.9 ± 0.7
fishy odour (P=0.08)	1.0 ± 1.0	1.1 ± 0.6	0.3 ± 0.2	0.6 ± 0.3
rancid odour (P=0.12)	0.8 ± 0.8	0.6 ± 0.6	0.3 ± 0.2	0.6 ± 0.5
chicken flavour (P=0.28)	6.8 ± 1.1	6.7 ± 0.6	7.3 ± 0.8	6.8 ± 0.7
fishy flavour (P=0.27)	0.9 ± 1.1	1.0 ± 0.7	0.3 ± 0.2	0.5 ± 0.6
rancid flavour (P=0.20)	0.5 ± 0.6	0.7 ± 0.7	0.3 ± 0.4	0.4 ± 0.4
overall impression (P=0.23)	3.6 ± 1.0	3.4 ± 0.7	4.1 ± 0.4	3.7 ± 0.5

*mean and standard deviation of 4 replications

CONCLUSIONS

The research proved that dietary treatments in the 1st experiment resulted in significant deposition of n-3 PUFA in white and dark chicken meat, which improved the n-6 PUFA/ n-3 PUFA ratio. At the same time, according to sensory analysis, weak, yet still noticeable fishy odour and flavour was noticed in all samples, but statistically significant difference was found only for fishy flavour in dark meat (P<0.05). Involvement of PBE1 and PBE2 oils in dietary treatments in the amount of 0.25% (A and B) and 0.5% (C and D) had positive influence on deposition of EPA and DHA in chicken meat, however it caused poorer sensory characteristics of meat.

Results of the 2nd experiment proved that modification of diet content with respect to concentration and sources of oils can result in production of chicken meat enriched with n-3 PUFA having satisfactory sensory characteristics.

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SIGNIFICANCE OF CARP (*Cyprinus carpio*) FEEDING WITH CHIRONOMIDAE LARVAE FOR MEAT QUALITY IMPROVEMENT IN THE SEMIINTENSIVE PRODUCTION SYSTEM

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ABSTRACT: Fish nutrition based on combination of natural food (zooplankton and bottom fauna) as main protein source, and added food, mostly cereals, as energy supply, is one of the main features of the world prevailing, semi-intensive farming system. In the recent years in several countries of South East Europe instead of cereals pelleted and extruded feed is used in carp nutrition in periods of depression of natural food. This is the way to overcome the lack of proteins and lipids, of required amino and fatty acids composition.
Bottom fauna organisms represent natural food of greatest importance for fry and cultured consumable carp. Because of its mass development in spring and lesser in autumn, Chironomidae larvae are particularly rich source of nutrients for carp. They are an easily noticeable pray because of their body dimensions (till several centimeters) and clear red color (coming from hemoglobin). Chironomidae larvae are invaluable protein source of adequate amino acid composition for carp body growth. Also, this is a source of lipids, particularly rich in omega 3 and omega 6 fatty acids. High omega 3 fatty acid content is present in Chironomidae, especially in *Chironomus plumosus,* the most frequently observed species in fish ponds. In *Chironomus plumosus* larvae, omega 6 fatty acids content compared to omega 3 content is higher. This is of particular importance providing higher needs in omega 6 for carp.
Considering that omega 3 and omega 6 fatty acids ratio in farmed fish is lower compared to natural populations; that the consumption of farmed fish is increasing together with requirements to increase omega 3 content, and optimally balance omega 3/omega 6 ratio; research in carp meat improvement are of utmost importance. Stimulation of natural food development particularly Chironomidae larvae in carp ponds is one of the approaches for enhancement of farmed carp meat quality.
Keywords*: Chironomidae, bottom fauna, carp, semiintensive farming system, meat quality*

INTRODUCTION

The most commonly represented carp production system is semiintensive (Tacon and De Silva, 1997). It is based on combining natural food as main protein source and added feed as main energy source. Due to relatively low prices and availability, cereals are the most frequently used added feed. Since carbohydrates make the largest part of cereals, the carp converts it into fat (Kaushik, 1995), making it less attractive for human consumption. In order to decrease the fat content of carp, but also to overcome the natural food deficit in the summer period, in several Easter European countries (Serbia, Bulgaria, Romania, and Bosnia Herzegovina) instead of cereals, pelleted and extruded concentrated feed is increasingly used. While employing cereals, carp meat quality can be improved by stimulating natural food development. When using concentrated feed, in order to decrease production costs, natural pond potential is maximally exploited. Obviously in both cases natural food is an important source of nutrients for carp. One of the central components of natural food are larvae of insects from the family Chironomidae. In some part of the season they represent a dominating component of carp natural food. As inestimable protein source of adequate amino acid content for building carp body, as well as source of lipids, primarily

omega 3 and omega 6 fatty acids, an increased attention is directed towards these organisms.

NATURAL FOOD AND ITS SIGNIFICANCE IN CARP PRODUCTION

In the semiintensive system carp natural food primarily consists of zooplankton organisms (Rotatoria, Cladocera and Copepoda) and bottom fauna (Chironomidae and Oligocheata). Carp consumes also macrophyte grains and young plant buds. The significance of zooplankton and bottom fauna organisms for carp rearing is primarily their nutritive composition. These organisms are crucial source of amino acids, lipids, fatty acids, vitamins, and enzymes for all age categories of cultured carp. Lack of natural food results in deficiency of vitamins, minerals and essential amino and fatty acids. This reduces fish diseases resistance and health condition, and even increases diseases frequency and mortality (Marković, 2010).
From the natural food, zooplankton and bottom fauna, carp obtains building components – proteins. Protein dry matter content in zooplankton organism represents over 40 % (Daphnia – 46.6%, Copepoda 41.6%), while in bottom fauna organisms proteins occupy over 50% (Chironomidae – 55.2%, Tubificidae - 54% proteina, Marković, 2010). Carp starts consuming natural food intensively as early as at 5 – 6 ^{o}C. In the beginning of the growing season zooplankton makes 60 % of natural food and bottom fauna 40%, while at the end of the growing season this ratio changes in favor of bottom fauna, except in cases when bottom fauna is exhausted - than fish switches again to zooplankton consumption (Marković, 2010).
In fish ponds natural food expresses significant seasonality in terms of development maxima of particular species: Rotatoria have their maximum in spring period, Cladocera end spring - beginning of summer, and Copepoda are the most abundant in autumn. Generally, zooplankton biomass is the highest in spring, in May, when it can reach several tons per hectare (Marković, 2010; Dulić et al., 2011). Highest biomass values for Chironomidae larvae is in the beginning of spring, in April-May, although sometimes in carp ponds there is a second maximum (much lower than the spring one) at the end of the summer period (second half of August, first half of September). Oligochaeta are the most abundant in summer.

Bottom fauna

In productive fish ponds bottom fauna is represented by insects' larvae – Chironomidae and annelid worms – Oligochaeta. These organisms are a very important natural food for carp fry and cultured consumable carp. A known fact is that in the first months of life carp consumes zooplankton, while adults prefer feeding on bottom fauna (Horvath et al., 2002). In order to make natural food available to the fish during the whole production season, it is necessary to stimulate natural food development in fish ponds by agrotechnical measures.
Dynamics of bottom fauna is influenced by fish stocking density, therefore high stocking density deplete bottom fauna. Its abundance is the effect of a range of factors, particularly: fertilization, age of the pond, physical and chemical properties of the water, to mention the most important.
In carp semiintensive production the yield on the farm, the quantity of added feed used, as well as the health status, and therefore a profitable and stable production depend on the quantity and composition (quality) of the available natural food.

Chironomidae

Chironomidae adults lay eggs in the water, where later larvae emerge. Larvae C*hironomidae* are dominant in many shallow lentic habitats, carp ponds included (Broza et al., 2000). Larval stage consists of four stages. At the end of each, a chitin head capsule is rejected. Larval stage, depending of water temperature, lasts for several weeks to a year. They feed on green algae and detritus. At the end of the fourth stage larvae becomes pupae (pupation), and later adults that do not feed appear. In the stages of pupae and adult these organisms live only few days.

Larvae Chironomidae are a widely distributed group of aquatic invertebrates that inhabits different types of water reservoirs. Their mass development in spring and lesser in autumn represents a valuable source of nutrients for carp (Marković, 2010). These larvae are an easily noticeable pray because of their body dimensions (till several centimeters) and clear red color coming from the respiratory pigment hemoglobin. Their body movements are distinctive "S" shaped wriggling, thus the carp reacting to pray motion can find and eat it easily.
In fish feeding experiments Chironomidae larvae usually serve as control feed, since growth and weight gain results obtained with these larvae are comparable to the results obtained using concentrated feed (Wolnicki et al., 2006).

FACTORS AFFECTING FISH MEAT QUALITY

In addition to its content of biologically important proteins, minerals and vitamins, fish meat is one of fundamental sources of essential fatty acids in human nutrition. Particularly important are omega 3 and omega 6 fatty acids that reduce level of triglycerides and cholesterol in serum (Steffens, 1997), lower the risk from coronary diseases (Wang et al., 2006), and hypertension (Berry and Hirsch, 1986). Lipids in fish contain more polyunsaturated fatty acids (*PUFA*), particularly omega 3 (such as alpha-linolenic acid, eicosapentaenoic acid – *EPA*, and docosahexaenoic acid - *DHA*) compared to warm animals meat (beef, pork, sheep meat) and vegetal oils (Ackman, 2000; Abbas et al., 2009; DeFilippis et al., 2010). In human body omega 3 fatty acids (*EPA* and *DHA*) synthesis from precursors of plant origin is not sufficient, therefore their intake must be provided from the food (Williams and Burdge, 2006; Lecerf, 2007).
Lipid and fatty acid content in fish tissues depends on different factors: environmental conditions, feeding type, genetic predisposition (Łuczyńska et al., 2012). The quantity of essential fatty acids varies accordingly to: fish feeding type (herbivorous, omnivorous, carnivorous), whether the fish is wild or cultured, age of fish, origin and composition of feed (Steffens, 1997). Feeding period and season also affect fatty acid content (Guler et al. 2008). According to Mareš et al. (2009) blue green algae bloom has significant effect on individual fatty acid content in carp muscles. Besides salts dissolved in water, its temperature has a significant effect on fatty acid content in fish tissues. At low water temperatures unsaturated long-chain fatty acids concentration increases, i.e. omega 3/omega 6 fatty acids ratio decreases (Amal et al., 1990).
Investigation of fatty acids content in carp have shown that different production and feeding methods cause important variation in the omega 3 and omega 6 *PUFA*. Carp fed additionally with cereals contains lower quantities of omega 3 *PUFA* (Steffens and Wirth, 2007). omega 3/ omega 6 *PUFA* ratio in carp additionally fed commercial pelleted feed is very similar to omega 3/omega 6 ration in the feed. Content and type of lipids in the feed affect content and quality of lipids in carp meat. Numerous research point out that fatty acid content in feed affects fatty acid content in fish meat (Steffens and Wirth, 2007). Feed rich in omega 3 *PUFA*, under the same breeding conditions, markedly increases omega 3/ omega 6 *PUFA* ratio in fish tissues.
Carp is less sensitive to essential fatty acid deficit than fish from the Salmonidae family. It has been found that 0.5 % of highly unsaturated fatty acids (*HUFA*) in carp feed is more effective than 1 % essential linoleic fatty acid (Watanabe et al., 1975).
The best weight gain and feed utilization is reached by combining 1 % linolenic and 1% linoleic acid in carp feed (Csengeri et al., 1978). This combination of fatty acids can be successfully replaced with 0.5 to 1 % omega 3 *HUFA* (*EPA* and *DHA*). Also, excessive quantity of essential fatty acids in feed for carp has as a consequence their decomposition and conversion in oleic acid, while lowering of fatty acid content below fish needs causes carp growth retardation and other disorders. Bogut and Opačak (1996) report most frequently observed symptoms of lack of essential fatty acids: fatty liver degeneration, myocarditis, decrease of hemoglobin concentration in the erythrocytes, increased breathing frequency, reduced feed utilization, retardation of growth, slow fin growth, increased stress tendency. On the other hand Kamler et al., 2008 reported that fin deformation in Cyprinids is the effect

of high concentration of omega 3 fatty acids (26.9% of total lipids belong to *DHA*) and lower concentration of omega 6 *PUFA* (4.9%) in the added feed. It has been demonstrated (Kamler et al., 2008) that by replacing commercial dry feed with natural one rich in omega 6 originating from Chironomidae (containing a total of 28.8% linolenic and arachidonic acid) that are poor in omega 3 (5.9% omega 3 *PUFA*), there is decrease in deformities of fish fry. It is recommended that commercial feed for fish fry should resemble as much as possible their natural invertebrate food (Kamler et al., 2008).

IMPORTANCE OF FEEDING ON CHIRONOMIDAE LARVAE FOR CARP MEAT QUALITY IMPROVEMENT

Among many sources of natural carp food, Chironomidae larvae occupy an important place. In stagnant aquatic ecosystems, because of its dominance (up to 80% of benthofauna biomass in stagnant water is this group of organisms – Janković, 1966) these larvae represent the irreplaceable link in the food chain by transporting energy towards higher trophic levels – fish. It has to be mentioned that proteins, amino and fatty acids usage from Chironomidae body depends on water temperature, dissolved oxygen concentration, and period of carp feeding with added feed.

According to literature data on fatty acid content of Chironomidae, content of saturated, monounsaturated and polyunsaturated fatty acids varies in our geographic region (North-East Croatia, central Serbia). Results of Živić et al., (2011) have pointed out higher content of saturated fatty acids in larvae *Chironomus plumosus* (45.36 i.e. 53.47%) compared to results of Bogut and co-workers (26.12%, Bogut et al., 2007). Unlike that, monounsaturated fatty acid content was somewhat lower – 28.11% (Živić et al., 2011) compared to results published by Bogut and collaborators (30.42%, Bogut et al., 2007), as well as *PUFA* content of 28.85 i.e. 16.38% (34.03%, Bogut et al., 2007).

High concentration of omega 3 fatty acids in Chironomidae, particularly in the species that is frequently found in fish ponds, *Ch. plumosus* (from 4.48 to 8.22% - Živić et al., 2011, to over 15.22% - Bogut et al., 2003), represents a supply of important fatty acids not only for carp, but for humans as well. From the omega 3 fatty acids in the lipid fraction of *Ch. plumosus* larvae, highly represented are linoleic – 7.21% (Bogut et al., 2007) i.e. 7.78% (Živić et al., 2011), *EPA* from 1.27% (Živić et al., 2011) to 4.36 % (Bogut et al., 2007) and *DHA* (2.49 %) (Bogut et al., 2007). High linolenic acid content is probably the consequence of green algae presence in phytobenthos that serves as food for *Ch. plumosus* (Sushchik et al., 2004).

Highest percentage of omega 6 fatty acids in larvae *Ch. plumosus* belongs to linoleic acid and varies from 13.76% to 21.37% (Bogut et al., 2003, 2007; Živić et al., 2011). *Ch. plumosus* larvae are richer in omega 6 than omega 3 fatty acids (from 18.81 - Bogut et al., 2003; to 22.31% - Živić et al., 2011). This is very important for cyprinid fish provided their higher needs in omega 6 than omega 3 fatty acids (Radünz-Neto et al., 1996). Established ratio of omega 6 and omega 3 fatty acids of 0.21 to 0.81% in the mentioned studies (Bogut et al., 2003, 2007, Živić et al., 2011) makes *Ch. plumosus* larvae excellent source of natural food for carp fry.

Having in mind the importance of Chironomidae in carp feeding, their development in fish ponds can be stimulated by using fertilisation as agrotechnical measure. Results of experiments performed imply increase in abundance and biomass of Chironomidae larvae when fertilization is applied. Chironomidae are actively cultured in Hong Kong by fertilizing a chosen field (before it turns into a pond) with chicken manure. Chironomidae adults lay eggs in water, so the lake becomes rich in these “blood worms”, they are easily collected and used as fish feed (Köksal et al., 1997).

CONCLUSION

Due to the following facts: in reared fish lower value of omega 3/omega 6 fatty acid ratio compared to this ratio in fish from natural habitats (Van Vliet and Katan, 1990), increased

consumption of cultured fish in human diet, tendency of consuming fish with high omega 3 content, and appropriate omega 3 / omega 6 ratio; research in improvement of fish meat quality are of utmost importance. This is confirmed by the studies realized. Among organisms that makes cultured carp natural meal, due to the rich content in *PUFA*, particularly omega 3 and omega 6, a family Chironomidae occupies an essential position.

ACKNOWLEDGEMENT

The present study was supported by the Serbian Ministry of Education and Science, through the project Improvement of production capacities of carp (*Cyprinus carpio)* through nutrition and selective breeding programs (TR 31075).

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THE INFLUENCE OF FEEDS ON FATTY ACID COMPOSITION OF MEAT FATTENING PIGS

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ABSTRACT: The aim of this study was to investigate the possibility of using different feed compound for pigs in order to increase content of n-3 fatty acids in adipose tissue, as well as improving relation n-6/n-3 fatty acid that is known to be disordered in the modern way peoples diet.
In this trial to view this crossbred pigs were used (Yorkshire x Landrace), with an initial body weight of 60 kg. The pigs were divided into two groups of 10 pigs and fed standard diets for fattening pigs of 60-100 kg (finisher), provided differences between groups only in the S experimental group which had a full-fat soybean in feed mixtures (S), and a second group enriched linseed (L) in the recommended amount of 2.5% in the feed mixture (Vitalan, Vitalac, France). Vitalan contained 85% of extruded linseed and the rest were wheat bran and antioxidants. The mixtures were balanced and fully meet the requirements of the animals at this stage of fattening. Samples were collected for testing of complete mixtures of chemical composition and fatty acid content. Pigs were kept until weight of 105 kg. At the end of the experiment, after slaughtering, processing and cooling carcass samples of adipose tissue (back) were taken of each pig in both groups. The adipose tissue was examined of fatty acid composition.
Chemical analysis of fatty acid composition (a gas chromatograph GC/MSD GC 6890, MS 5972/73) complete feed mixtures showed that the feed with linseed (L) had significantly lower ($P < 0.001$) content of SFA, and significantly higher content ($p<0.001$) PUFA from feed mixtures with full-fat soybean (S). Also the ratio of n-6/n-3 in feed mixture in L group (5.567) was lower compared to feed mixture in S group (11.00) which is statistically highly significant ($P<0.001$).
By analyzing the fatty acid composition of pigs fat statistically significant differences were found between experimental groups. In adipose tissue of pigs in the groups with linseed (L) in mixtures was achieved significantly ($p<0.001$) higher content of n-3 fatty acids, or 1.97% compared to 1.42% in groups receiving full-fat soybean (S) in feed mixtures, and significantly ($p<0.001$) lower content of n-6 fatty acids, or 20.09% compared to 24.91%. Ratio n-6/n-3 in adipose tissue for the L group was significantly lower ($p<0.001$) or 10.23 relative to the group S (17.74).
Key words: *pigs, feed, linseed, fatty acid*

INTRODUCTION

Numerous medical findings show that a significant role in development of cardiovascular and other chronic diseases in humans have relationship between the two groups of polyunsaturated fatty acids in the diet: omega-6 acids, whose main representative is linoleic acid (C18: 2 n-6) and omega-3 fatty acids, whose main representative is alpha linolenic acid (C18: 3 n-3).
Unsaturated fatty acids have mutually differences in chain length, number and position of double bonds in molecules. The abbreviation "ω-6" (or n-6) indicates that the first double bond in the molecule of fatty acid is at the sixth carbon atom, as for "ω-3 '(or n-3) indicates that the first double bond in the molecule of fatty acid is at the third carbon atom, counting from the methyl group. In the natural resources most often reportered unsaturated fatty acids belong to the ω-6, ω-3 and ω-9 series.
The human body can not synthesize essential fatty acids, but retains the ability to translate basic essential fatty acid ingested by food (linoleic and α-linolenic) in the so-called long-chain

polyunsaturated fatty acids with specific roles in the body. In these biotransformation are involved enzymes desaturase and elongase and through out their activities, as well as the amount of substrate, depends the intensity and efficiency of these reactions. Desaturases are the enzymes responsible for the introduction of new double bond in fatty acid chain. There is a competition for these enzymes among fatty acids of ω-3 and ω-6 series. Elongases are the enzymes responsible for fatty acid chain extension by two carbon atoms, that is for introduction of two new methylene group.

Numerous studies confirm that increased intake of ω-3 fatty acids can affect on decreasing the risk of heart disease and vascular disorders, but also alleviating symptoms and improving the clinical picture in some autoimmune and inflammatory disorders.

Interest in the role of polyunsaturated fatty acids have been launched by investigations in 70th of the last century, which have revealed a very low occurrence of vascular disease in the population of Greenland Eskimos, despite their diet rich in fat and based on marine mammals and fish whose food chain is based on algae and plankton rich in omega-3 polyunsaturated fatty acids. Similar observations were later confirmed by epidemiological studies in other populations with a similar diet, for example in coastal areas of Japan (Karolyi, D. 2007).

In the period 1927-1930. The Evans and Burr, pointed to a number of disorders caused by eating fat-free diets, and then was introduced (Burr and Burr, 1930) term essential fatty acids for all those fatty acids that the body can not synthesize and that must be taken through out food in order to avoid the appearance of deficiency symptoms. From that time until today does not stop the interest of scientists in chemistry, metabolism and health significance of fat and individual fatty acids, and special attention is paid to the fatty acids of ω-3 and ω-6 series (Šobajić, 2002).

In the pigs nutrition are used soybean, sunflower and other oilseeds that contain fatty acids of ω-3 series and fatty acids of ω-6 series (Baltic et al., 2011, Markovic et al., 2011).

Flax (*Linnum usitatissimum L*.) is one of the oldest cultivated crops in the world market and also one of the most economically important crops. Flax seed contains about 35 to 45% oil compared to the mass of dry material (Karleskind, 1996). More than 70% of this oil contains polyunsaturated fatty acids, primarily alpha-linolenic acid (ALA), an essential ω-3 fatty acids and linoleic acid (LA), essential ω-6 fatty acids.

It should be noted that flax meal contains the fatty acid profile that is similar if not identical, as flax seed, considering that the total oil content of the meal is variable depending on the method used for extraction of oil, which affects the energy value of this nutrient.

Because of the many potential benefits of ω-3 fatty acids in the diet, consumer demands increase for ω-3 enriched products. Recommendations for daily intake of ω-3 fatty acid throughout diet, for adult men are 1.6 g, and for adult women 1.1 g (National Academy of Sciences, 2005).

A large number of products are now produced with the aim of enriching with ω-3 fatty acids. These products include ω-3 enriched eggs, bread, pasta, dairy products, baby food, milk, cereals juices, salad and meat dressings, and they are all available to purchase at most stores.

The ability of producing ω-3 enriched meat products of pigs is very interesting for many producers and consumers, as noted above. In monogastric animals such as pigs, fatty acids are absorbed from the gastrointestinal tract with small changes. In fact, the fatty acid profile of tissue directly reflects the profile of fatty acids in animal nutrition. In ruminants, however, the fatty acids from food in the digestive tract are changed under the influence of the process of microbial fermentation and biohidrogenation, prior to absorption from the gastrointestinal tract (Baltic et al., 2011b, Markovic et al., 2011b).

In modern human nutrition ratio of ω-6: ω-3 polyunsaturated fatty acids is relatively wide (10-15:1), so that today in human diet are made efforts to enrich food with ω-3 fatty acids, so that this relationship could be approximate to optimal (4:1).

MATERIALS AND METHODS

For the experiment were used crossbreed pigs Yorkshire x Landrace, with initial body weight of 60 kg. The pigs were divided into two experimental groups of 10 pigs and were fed with standard mixture (NRC, 1998) for the final fattening pigs of 60-100 kg (finisher), with the differences between groups only in that the I experimental group, a group (S) received a soybean meal in the diet, and Group II received a preparation of linseed (L) in the recommended rate of 2.5% in the mixture (Vital-Vitalac, France).
The Vitalan preparation of flax seed contains 85% of extruded flax seed, and the rest of the preparation are wheat bran and antioxidants.
The mixtures were balanced and fully met the demands of the animals at this stage of fattening.

Table 1. Ingredients and chemical composition of swine mixtures, %

	S-group	L-group
Nutrients	%	%
Corn	44.5	55.7
Wheat, hard	25	14.5
Soya bean,roasted	14. 03	-
Soya bean, meal	-	9.3
Wheat, flour	-	10.0
Wheat bran	12.9	-
Corn gluten	-	4.7
Flax preparation	-	2.5
Di-Ca-P	0.6	0.6
Chalk	1.3	1.3
Salt	0.4	0.4
VMD	1	1
Σ	100	100
The chemical composition		
Moisture	11.36	11.83
Ash	4.86	4.42
Protein	14.52	14.59
Fat	5.30	3.87
Cellulose	4.22	2.95
Ca	0.72	0.72
P	0.61	0.46
ME - S	13.36	13.33
Lys	0.63	0.54
Met +Cyst	0.48	0.47

Samples were taken from the complete mixtures for the determination of chemical and fatty acids composition. At the end of the experiment, after slaughtering, processing and cooling carcass samples of adipose tissue (back) were taken from each pig in both groups. In adipose tissue fatty acid composition was examined.
Total lipids for fatty acid determination were extracted from pigs mixtures as for pork fat tisue with hexane / isopropanol mixture by accelerated solvent extraction (ASE 200, Dionex, Germany). After evaporation of solvent until dryness under the stream of nitrogen, total lipids were converted to fatty acid methyl esters (FAME) by trimethylsulfonium hydroxide. Fames were determined by using Shimadzu 2010 gas chromatograph equipped with flame ionization detector (FID) and cianopropyl HP-88 capillary column (100m x 0.25 mm x 0.20μm) (Spirić et al., 2010).

RESULTS AND DISCUSSION

In this study were investigated the amount and relations of fatty acids in pigs adipose tissue depending on the fatty acid content in the feed that were a part of mixtures for swine diet. From the results of the total fatty acid content in the feeds that were used (Table 2) we see that there were significant differences between complete feed mixtures for these groups. Complete feed mixture of L group, that received preparation with extruded linseed had a significantly lower content of SFA and significantly higher content of PUFA ($p < 0.001$) compared to S group. The trials showed that more than 70% of flax seed oil consists of polyunsaturated fatty acids (mainly alpha-linolenic acid (ALA) and linoleic acid (LA) (Nikolovski et al., 2008).

Table 2. Fatty acids in the feed – total content

Group	SFA (%)		MUFA (%)		PUFA (%)	
	$\bar{X}$	Sd	$\bar{X}$	Sd	$\bar{X}$	Sd
S	20,17$^{\alpha}$	0,32	25,51	0,32	54,33$^{\alpha}$	0,48
L	18,38$^{\beta}$	0,60	25,46	0,26	55,99^{ß}	0,70

Different letters α, ß denote significance of $p < 0.001$ (Table 2-5)

Table 3 presents content of n-6, n-3 fatty acids and their ratio n-6: n-3. Complete mixture for swine, which is in its composition contained flax preparation, had a significantly higher ($p<0.001$) content of n-6 and n-3 fatty acids, and their ratio n-6: n-3 was also significantly lower (5.57) compared to the total mixture without flax preparation (11.00).

Table 3. The total content of n-6, n-3 and ratio of n-6: n-3 fatty acids in the feed

Group	n-6 (%)		n-3 (%)		n-6:n-3	
	$\bar{X}$	Sd	$\bar{X}$	Sd	$\bar{X}$	Sd
S	49,79$^{\alpha}$	0,41	4,54$^{\alpha}$	0,22	11,00$^{\alpha}$	0,54
L	47,46$^{\beta}$	0,60	8,53$^{\beta}$	0,22	5,57$^{\beta}$	0,14

Fatty acid profile of the carcass directly reflects the profile of fatty acids in the diet of animals (Eastwood, 2002). Since flax seed has desirable fatty acid composition, many producers are interested in including it in the finisher pig diets and therby improve the fatty acid composition of meat.

In this experiment, the total content of SFA and MUFA of fatty acids was significantly higher ($p < 0.001$) in adipose tissue of pigs that were fed with diet that contained flax, compared to another group of pigs, but the amount of PUFA in the adipose tissue of those pigs was significantly lower ($p < 0.001$) (Table 4).

Table 4. Fatty acids in adipose tissue - total content

Group	SFA (%)		MUFA (%)		PUFA (%)	
	$\bar{X}$	Sd	$\bar{X}$	Sd	$\bar{X}$	Sd
S	31,30$^{\alpha}$	0,52	41,91$^{\alpha}$	0,48	26,32$^{\alpha}$	0,40
L	33,16$^{\beta}$	0,51	44,42$^{\beta}$	0,26	22,05$^{\beta}$	0,46

One of the first studies that examined effects of feeding with flax seed on lipid profile of pigs carcass was performed by Cunnane et al. (1990). The pigs were fed with diets containing 5 per cent of flax aged from 2 weeks unil 10 weeks of age. Pigs had significantly higher level of ALA in their liver, kidney, heart, skin, subcutaneous adipose tissue and muscle, and significantly higher level of DHA and EPA in the liver, kidneys and heart. This study provides evidence that including of Flax seed improves the omega-3 fatty acids profile of pigs carcasses.

After that, more research have been conducted in order to determine the optimal level of inclusion of flax seed and appropriate duration of feeding flax seed to ensure the enrichment of omega-3 fatty acids, with no negative impact on carcass quality. Romans et al. (1995 a,b) conducted two trials in order to acquire answers to these questions. In the first study, pigs were fed with diets containing 0, 5, 10 or 15% flax seed 25 days before slaughter. Researchers have obtained a linear increase of ALA in the back fat and bacon of animals. The levels of ALA in the pigs back fat were 10, 23, 37 and 53 mg/g tissue with inclusion of flax seed at 0, 5, 10 and 15% respectively. The authors concluded that flax seed should not be used in finisher for pigs above the level of 15% due to changing sensory properties of meat.

In another study of Romans et al. (1995 b), the pigs were fed with a level of 15 percent of flax seed, but the duration of treatment varied. The pigs were fed during 7, 14, 21 or 28 days before slaughter. ALA content of adipose tissue back (backfat) increased significantly linearly with increasing duration of using allowancse with flax seed. It is also important to note that the allowances used in two studies Romans et al. (1995a, b) were based on corn. Since corn is rich in omega-6 fatty acids (linoleic), the effect of inclusion of flax seed will not be as good as when allowances with wheat and barley. Fontanillas et al. (1998), Matthews et al. (2000), Enser et al. (2000) and Thacker et al. (2004) are just some of the studies that show similar effects of feeding with flax seed on fatty acid profile in pigs carcasses obtained from finishing pigs.

Application of flax seed preparation (group L) led to significantly ($p < 0.001$) lower (20:09) total content of n-6 fatty acids in adipose tissue, and significantly ($p < 0.001$) higher (1.97) content of n-3 fatty acids compared to the group of pigs (S) that did not receive this preparation (Table 5). Also the ratio n-6: n-3 in the experimental group L was significantly lower (10.23) compared to S group (17.74)

Table 5. The total content of n-6, n-3 and ratio of n-6: n-3 fatty acids in adipose tissue of pigs

Grupa	n-6 (%)		n-3 (%)		n-6:n-3	
	$\bar{X}$	Sd	$\bar{X}$	Sd	$\bar{X}$	Sd
S	24,91 $^{\alpha}$	0,43	1,42$^{\alpha}$	0,13	17,74 $^{\alpha}$	1,89
L	20,09 $^{\beta}$	0,47	1,97$^{\beta}$	0,09	10,23 $^{\beta}$	0,57

Okanović et al. (2010) have been determing the impact of food enriched with flax seed on the omega-3 fatty acids content in swine until the mean weight of 110 kg. Treatment with feed that contains flax seed resulted in higher concentration of omega-3 fatty acids (>7 mg/100 g) which reduced the ratio of omega-6 and omega-3 fatty acids in meat (<3) making it, from a health perspective, the better for human nutrition.

Stanislawa Raj et al. (2010) used in their trial gilts weight 60-105 kg divided into four groups (control group, group with linseed oil - L, rapeseed oil - R, beef tallow -T and with fish oil - F). All groups received allowances that were balanced in energy but with different ratios of PUFA and SFA, and linolenic acid (C18: 2n-6) to α-linolenic acid (C18: 3n-3). The linear relationship was established between the intake and utilization of C18: 2n-6 and C18: 3n-3 in the body. Efficiency of utilization of C18: 2n-6 was lower than C18: 3n-3.

Vaclavkova and Beck (2007) have examined the effect of different contents of flax seed on meat quality of pigs with body weight 37-98 kg. The pigs were divided into four groups: control (group with no flax in the diet -LO, group with 6.7% flax seed - L1, group with 13.4% of flax seed -L2, and group with 13.4% flax seed with the addition of 103 mg α-tocopherol - L3). Adding flax seed into the allowance for pigs significantly ($p < 0.05$), increased the content of linoleic acid, α-linolenic ($p < 0.001$), arachidonic ($p < 0.05$) and eicosapentaenoic (EPA) acids ($p < 0.05$) but had no effect ($p > 0.05$) on the content of docosahexaenoic (DHA) acids in muscle tissue. The ratio n-6: n-3 was lower in the experimental groups, but not significantly ($p > 0.05$). The total content of n-6 and n-3 fatty acids was the lowest in the control group.

In trial conducted by Mourot and Hermier., 2001, were compared effects of the three allowances (supplemented with beef tallow, with rapeseed or with flax seed oil) on fatty acid composition of broilers and pigs muscle. Among these three allowances, diet supplemented with flax seed oil led to the highest content of N-3 PUFA in the pigs and broilers muscles, and the lowest n-6/n-3 ratio. Kouba et al., (2003) in his trial examined total fatty acid content in *M.longissimuss* of pigs fed with 6% flax seed in the allowances during 20, 60 and 100 days. They have determined a significant reduction of n-6: n-3 ratio in these groups, and increased PUFA / SFA compared to the control group.

CONCLUSION

Diet enriched with extruded linseed had a beneficial impact on the content of n-3, n-6 acids and the ratio of n-6: n-3, which is significant for the health of consumers.

ACKNOWLEDGEMENTS

The experiment and researches have been accomplished within the Project TR 31034 "Selected biological hazards to the safety/quality of food of animal origin and the control measures from farm to consumer", funded by the Ministry of education and science of Government of Republic of Serbia.

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BIOLOGICAL TREATMENT OF ANIMAL FEEDS

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ABSTRACT: Biological treatment of feeds includes its exposure to the influence of exogenous enzymes in digestive tract of animals, i.e. exposure to the process of fermentation under the influence of microorganisms (pre-digestion) in fementors. Basic reason for adding endogenous enzymes into animal diets is the activity of already existing unused nutrients into outer space. The technology of pre digestion of food includes exposure of food to the process of action of added enzymes in fementors which enable undisturbed and successful fermentation. Microorganisms isolated from rumen of ruminants dissolve cellulose, hemicellulose, lignin and other difficultly digestible polysaccharides using their own enzymes, and the products of dissolution (simple sugars) are used for the synthesis of microbial protein of high biological value. They use nitrate and nitrite nitrogen from food and from the air. They synthesize vitamins of B complex, D, E, and K vitamins and they also dissolve mycotoxines, making the food contaminated with mycotoxines less harmful. Through the process of fermentation of feeds, under the influence of microorganisms of rumen ("Biofermix" preparation) examined in Russian scientific research institutes, in cases of certain animal feeds (e.g. wheat bran), the increase in the protein content was determined from 13 to 25%, as well as reduced content of cellulose from 8 to 2%, which contributes to the greater utilization of such feed, to the better production reproductive and health performances of animals and to the protection of the environment as well. In order to gain new experience and to obtain additional arguments on efficacy of utilization of microorganisms in the process of biological treatment of animal feed it is necessary to perform research work with raw materials which is used under our conditions.
Key words: *Animal feed, microorganisms, enzymes, fermentation*

INTRODUCTION

Newly formed genotypes of highly productive animals demand greater amounts of quality feed, which points out to the need of rational usage of all available resources for its production. Especially important segment in this plan is rational usage of inadequately used resources, first of all by-products from plant production and food industry. Such resources of feeds are mostly in the form or in the shape which does not allow simple or immediate use in animal feeding. Therefore, it is necessary to prepare it for storage and for later usage. Different procedures for food processing are available nowadays and they could be classified as physical, chemical and biological procedures. Physical procedures include chopping or mixing, water treatment, cooking under pressure or without pressure and exposure to gamma rays. Chemical procedures include exposure of feeds to different compounds such as: sulfur dioxide, sodium hydroxide, alkaline hydrogen peroxide, potassium hydroxide, ammonia, ammonia hydroxide or ammonia bicarbonate, and urea and calcium hydroxide (Grubić et al., 1995). The aim of such treatment is to break or to weaken lignocellulosic connections in feed and to increase the area of the effect of digestion process of enzymes of endogenous or exogenous origin. Biological treatment includes exposure of feed to the effects of enzymes brought into the body with feed (mixed into feed), i.e. exposure of feed in solid state of the fermentation process in fermentors in the presence of certain microorganisms (Nasseri et al., 2011; Irfan et al., 2011; Popov 2007; Grujić et al., 1995).

Lynd et al., (2002) claim that, since the time the processes of enzyme conversion of celluloses were determined, dominant factors, which refer to human benefits and which became sustainable sources of energy certainly provide energy and global climate changes. These significant influences increased the need to pay greater attention to using these sources of energy. Great efforts were invested into examinations and development of practical processes for conversion of celluloses into energy and substances which could be of usage. The most important aspect of such processes implies decomposition of cellulose using microorganisms.

DEVELOPMENT AND BASIC CHARACTERISTICS OF BIOLOGICAL TREATMENT OF ANIMAL FEEDS

Development and basic characteristics of animal feeds through exposure to enzymes started during sixties of the last century. Enzymes are substances of protein nature which decompose feed to simple compounds that are suitable for adsorption, but they also enable a whole line of metabolic processes. The basic reason for adding of exogenous enzymes into animal feeds is to supplement the activity of endogenous enzymes of animals, that is, acceleration of feed into forms which are available for the resorption of nutrients and for reduction of excretion of unused matters into environment. Enzymes used for this purpose are produced by modern biotechnological procedures via controlled fermentation of substrates of chosen strains of fungi (e.g. *Thricoderma viridae, Aspergillus oryzae and Aspergillus niger*) and bacteria (*Bacillus subtilis, Bacillus licheniformis* and other). Isolated microorganisms excrete the enzyme into medium which is then filtered, purified and then put into appropriate carrier (powdered or fluid) depending on the equipment for their application in factories for animal feed production. It first started by adding separate enzymes (protease and amylase). In later stages it was shifted to application of adding enzyme preparations which contained more enzymes. Enzymes were added into feed during mixing in small amounts (under 1%), which means they did not require special equipment for application. Lately, enzymes are produced in liquid form and they demand appropriate equipment for application, but their efficacy is greater. They are added into feed after pelleting or extruding, which excludes a possibility for their exposure to high temperatures and damages. The greatest need and justification for using enzymes is in feeding young animals, especially pigs and chicken, because the enzyme systems of their gastrointestinal tract is underdeveloped (Sinovec, 2003). Especially interesting enzymes are hemicellulases, cellulases, pectinases, beta-glucanases; xylanase and alfa-glucosidase, which decompose structural carbohydrates (plant cell walls). Using enzyme preparations in feeding pigs and poultry has a positive effect to the health of animals and their production results (growth, intake and feed conversion). Described effects are especially expressed in broilers fed on grains (barley, wheat, and oats) and plant protein feeds (sunflower meal, peanuts meal and rapeseed meal). Application of enzymes in hen feeding has positive influence on laying and quality of eggs, thickness and firmness of egg shell (Cmiljanić et al., 2007). Using enzyme preparations which contain a cocktail of enzymes in meals for poultry with greater content of non-starch polysaccharides at the same time makes better usage of greater number of nutrients (Lević and Sredanović, 1999; Steen 2003; Hryby 2004; Slomijski 2005; Cmiljanić et al., 2006). During the last years the enzyme phytase is used in order to decompose phytic acid and release of phosphorus in substrate (feed) which reduces the need for adding inorganic phosphorus in animal meals (Jongbloed et al., 2000; Mellor, S. 2004). This is especially important because 50-80% of phosphorus is tied to phytic acid (Simons et al., 1990; Šefer and Sinovec, 1998). Considering localization of phytates in aleuronic layer of plant material, a combined usage of phytase with cellulolytic enzymes, such as beta-glucanases and pentozonase, is recommended.

When using enzymes in cattle feeding, one should keep in mind they fall under the influence of enzymes of microorganisms of the rumen which decompose them and make them less active. Three groups of microorganisms are present in the rumen of ruminants: bacteria,

protozoa and fungi. One group of these microorganisms decomposes nutrients brought into the rumen by feed, and the other group uses the products of their decomposition.
In fattening calves, proteases and amylases could be used, especially in cases of early weaning of calves (less than 6 weeks of age) when it is necessary to provide them with feed of high digestibility and high energy concentration. Dawson (1999) proved that exogenous enzymes (cellulases and xylanases) influence increase of digestibility of dry matter and NDF (neutral detergent fiber), higher production of evaporable fatty acids was also proved with the improvement of ratio of acetic and propionic acid and the increase of pH value of the content of rumen. The same author also states that milk yield of cows increased for 0.82-2.8 kg when using protected enzymes. Appropriate biotechnological procedures which enable the protection of exogenous enzymes from the influence of rumen microorganisms are nowadays being developed in the world. The procedure is based on the principle of glycolysis, used in the nature by fungi which excrete secretion and in that way protect their enzymes from the environmental proteases in which they develop. Zinn (1999) established that application of protected enzymes in feeding fattening calves contributes to increasing of digestibility of organic matter from 61 to 63.2% and NDF from 28.22 to 34.7% with the increase in growth from 1.33 to 1.41 kg/day and to reduction of feed per kg of growth for 1.89%. Protected cellulolytic enzymes, apart from positive effect on the consumption and efficacy of utilization of feed and production performances of animals cannot be adequate replacement for poor quality of forages and poor management in its production (Howes, 1996). Conditions that an enzyme has to fulfill are: expressing its activity at an optimal pH value of the environment, resistance to endogenous proteolytic enzymes and low pH of the stomach, stability during storage and thermo stability. Enzymes can influence the reduction of unwanted effects of certain anti nutritive matters (beta glucans, arabinoxylans, pectins, galaktosides, inhibitors of proteases and amylases, tannins, lectins, glycosides, phytates, alkaloids and other) in feed such as soya, lupine, millet, rapeseed and other.

PRODUCTION AND UTILIZATION OF MICROBIAL PROTEIN

Thanks to the modern biotechnological achievements nowadays it is possible to produce microbial origin, so called single cell protein (SCP). For the microbiological synthesis of proteins (Bhala et al., 2007) use bacteria (*Cellulomonas sp. Bacillus subtilis, Aeromonas hydrophylla* and other), yeasts (*Candida utilis, Saccharomyces cereviciae, Amoco torula and other*), fungi (*Thrichoderma viridae, Aspergillus niger, Aspergilus oryaze, and other*) and algae (*Spirulina sp., Chlorella pyrenoidosa, Scendesmus sp.* and other). These microorganisms in a short time interval of only few hours can multiply the quantity of protein with very favorable amino acid content, bacteria for 0.5 – 2 hrs, yeasts 1-3 hrs, algae for 2 – 6 hrs. Protein production is performed in fermentors on different substrates under aerobic conditions (except for algae), under the controlled temperature and pH value. By-products of agricultural production and food industry (sugar factories, starch, beer, dairy plants, wood manufacture, fruits and meat) are used as a substrate for their cultivation by the procedure of feed in solid state fermentation. At the same time, apart from protein production, this contributes to the solution of the problems of environmental pollution. For microbial bio-mass protein production under slightly different and more complex biotechnological conditions and by different procedures, natural gas, n-alkanes, paraffin, gas oil, methanol, ethanol can also be used (Đorđević and Dinić**,** 2007; Popov 2007). Chemical content of microbial biomass, (Nasseri et al., 2011) is given in Table 1.

Table 1.Chemical contents of biomass of different microorganisms (% dry matter)

Indicator	Fungi	Algae	Yeasts	Bacteria
Protein	30-45	40-60	45-55	50-65
Fat	2-8	7-20	2-6	1-3
Ash	9-14	8-10	5-10	3-7
Nucleic acid	7-10	3-8	6-12	8-12

Similar example of protein production in nature is oyster mushroom *(Pleurotus ostreatus),* which grows on substrate rich in cellulose, hemicelluloses and lignin (straw of wheat, soya, sawdust, bark of a tree, and other). Using its enzymes, this mushroom decomposes lignocellulosic ingredients up to simple compounds which, including nitrogen from the air, uses for feed and for the production of fruitful body.
Undissolved and dissolved residues which the mushroom did not use, as well as mycelium (hyphae made of chitin macromolecules) can be used, in limited amounts, for feeding of animals (Adamović et al., 1998). Grujić et al., (1995), investigated a possibility of protein production on a substrate made of hydrothermically treated wheat straw and cornstalk using cellulolytic moulds *Chaetomium cellulolyticum and* Chaetomium *globosum* by the solid state fermentation of feed. The results obtained showed that in case of mould *Chaetomium cellulolyticum* during five days of fermentation under aerobic conditions, the quantity of protein was increased nearly 100% (Table 2).

Table 2. Efficiency of Chaetomium *cellulolyticum* in the production of protein of microbial origin

Parameter	Start	End	Index
Wheat straw			
Protein,% DM	6.0	11.5	192
Cellulose,% DM	49.0	36.0	73
Cornstalk			
Protein,% DM	5.5	13.5	245
Cellulose,% DM	44.0	32.0	72

Similar results of fermentation of hydrothermal treatment of wheat straw and corn stalk were obtained when using *Chaetomium globosum.*

UTILIZATION OF «BIOFERMIKS» PREPARATION IN ANIMAL FEEDING

The scientists of the Moscow Agricultural Academy “K.A. Timirjazev” Russia, worked on the development of “Biofemiks” preparation, approved by the Pharmacology Council of the Ministry for agriculture and food of Russia (TU 9337-001-46391307-98). According to the given description, the preparation is in powder form and contains more than 40 species of microorganisms, first of all bacteria and fungi isolated from the rumen content of deer species moose. These microorganisms through their enzymes in by-products of agricultural and food industry (straw, corn stalk, husk, cob, chaff, wheat bran, raw brewer’s grains, sugar beet pulp and other similar products of plant origin) decompose celluloses, hemicelluloses, lignin and other difficultly digestible polysaccharides. In that process, microorganisms use the products of degradation (simple sugars), as well as nitrogen originating from nitrates and nitrites, including nitrogen from the air, and they synthesize microbial protein of high biological value. At the same time, they can use nitrogen from urea, nitrogen phosphates and diamonium phosphate. They synthesize vitamins of B complex, vitamins D, E and K. These enzymes also decompose mycotoxins, making the feed contaminated with mycotoxins less harmful.
Apart from microorganisms, “Biofermiks” enzyme preparation contains extracts of active substances of many herbs of high biological value. “Biofermiks“ is used in the amount of 5 g/1000 kg fermented feed. The mentioned amount is mixed with 3-4 kg of corn meal or wheat bran, and 3-4 L of warm water is added. Everything is mixture and let for 3-4 hrs at 20-30 ºC. In such a way prepared mass is mixed in fermentor with fragmented feed moistened with warm water (humidity 55-65%) and kept at 50-55 °C.
The length of fermentation depends on the content of cellulose in treated material and it ranges from 3-4 hrs for the level of cellulose of 5-8%, to 10-12 hrs for the level of 30-45%. Table 3 presents the results of the effect of preparation “Biofemiks“ on chemical content of wheat bran (Lesnov and Puzankov, 2006).

Table 3. Chemical content and nutritive value of wheat bran

Parameter	**Without „Biofermiks“**	**With „Biofermiks“**
Energy, MJ	8.8	10.5
Protein, %	13	25
Cellulose,%	8	2
NFE,%	52.6	50.2
Fat, %	4.1	4.2
Sugar, %	4.7	4.9
Calcium ,g/kg	1.80	1.80
Phosphorus ,g/kg	10.10	10.10
pH	6.5	6.5

It is obvious that the fermentation of wheat bran under the influence of “Biofermiks” preparation, as its authors stated, caused a great increase of the content of energy and proteins, i.e. the reduction of cellulose, which makes the results very interesting. The authors of this paper, however, are of the opinion that the confirmation of these results should be looked for in further investigations with the same or similar preparation. At the same time, they think that a full-scale evaluation of this preparation requires the results of their own research work.

In a trial performed by Savič Čahmahčev, (2000) the pigs in the experimental group were given 20% of mixture fermented with “Biofermiks “preparation and they had growth of 441 g/day, which was 31.6% greater than the growth of control group (335 g/day). For 1 kg of growth of the experimental group, 4.4 kg of fodder mixture was used and 5.5 kg for the control group. The feeding cost for one kg of growth in the experimental group was 20% smaller than in the control group. The pigs of the experimental group had the increase in the number of erythrocytes and the content of hemoglobin, glucoses and proteins. The increase of gamma globulins, albumins, alpha and beta globulins, calcium and phosphorus was observed which points out the positive effect of the preparation examined to the exchange of nutritive matters in pigs. The experimental group had a smaller number of incidences of illness of organs for digestion (16.3 : 40.6%). The same author found a positive effect of the preparation „Biofermiks“ in fattening pigs (Table 4) to the body mass, growth and for the content of proteins and fat in meat ($p<0.05$).

Table 4. Body mass, growth and physico-chemical content in pig meat

Indicator	**Without „Biofermiks“**	**With „Biofermiks“**
Body mass at the beginning of the trial	57.0	58.5
Body mass at the end of the trial	87.5	103.0
Growth, g/day	358	532
Chemical content of meat,%		
Humidity,%	69.38	71.41
Protein,%	19.11	22.28
Fat,%	3.24	4.07
Ash,%	0.92	0.86

In the second trial, the positive effect of “Biofermiks” preparation to the growth of fattening pigs was also established. The growth of experimental group (511 g/day) was greater than in the control group (449 g/day), for 13.8%, while the number of culled animals from fattening in the control group was greater for 13.3%.

In a trial performed on two groups with 50 cows in each group, (Lesnov, 2008) the effect of wheat bran fermented by preparation ”Biofermix” to the production of dairy cows was investigated. The experimental group of cows received 0.6 kg of fermented wheat bran (45% DM) during 25 days which is approximately equivalent to 0.35 kg of additional fodder mixture. Average milk yield of the experimental group of cows (on the 11^{th} day of the trial) was greater for 0.94 L or 4.40%, that is on 25^{th} day it was 1.86 L or 9.18%, which points toward appropriateness of using such technology in feeding lactating cows.

Based upon the data from the available literature, it could be concluded that most of the recommendations point to the limit of the quantity of 20-30% of microbial protein out of total proteins in diets of non ruminants. In diets for pigs, without any greater consequences, microbial protein could be substituted up to 55% of proteins of fish flour and soybean meal. Ruminants can use better microbial protein and the possibilities for substitution of conventional proteins are somewhat greater. There are data referring to sheep rations where these proteins could be used, without any greater consequences, up to 75% of protein in a diet. (http://www.fao.org/ag/AGA/agap/frg/ AFRIS/Data/734.htm)
When using proteins of microbial proteins there are certain limitations. One among the first is the content of nucleic acids. Metabolism of these acids increases the share of uric acid in urine and in blood serum. Uric acid, if present in greater amounts, creates kidney stones. Nowadays, however, there are methods and procedures (chemical and enzymatic) by which the reduction of nucleic acid could be influenced (Nasseri, 2011). Depending on the quality, there is a possibility for slower degradation of proteins in digestive tract, as well as certainty of occurrence of allergic reactions, contamination by pathogenic microorganisms and by toxic substances.

CONCLUSION

Use of contemporary biotechnological methods and procedures, based on the utilization of enzymes and microorganisms, can contribute to the more complete usage of insufficiently used feed resources. First of all by decomposing cellulose and improvement of protein quality, as well as the protection of the environment. In order to obtain necessary experience and additional arguments, it would be needed to perform further investigations and provide answers to other questions as well, particularly in the field of biological and economic justification of application of solutions discussed under our conditions.

ACKNOWLEDGEMENT

The work was realized within the projects III 46012 and III 46002 co-financed by the Ministry for Education and Science, Republic of Serbia.

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THE EFFECTS OF ANIMAL AND VEGETABLE FATS ADDED TO BROILERS' NUTRITION ON THE CHEMICAL CONTENTS OF MEAT AND OFFAL

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ABSTRACT: The purpose of this experiment was to establish the effects of the addition of animal and vegetable fats to broilers' nutrition on the chemical contents of red, white meat, liver and gizzard after 42 days of breeding. The Cobb 500 broiler was used in this experiment, in total 240 animals, placed in four separate treatment. In chickens' nutrition, there was increased content of fats by 3% (treatment I – lard; treatment II – soybean oil; treatment III – tallow; and treatment IV – sunflower oil). With analysis of chemical parameters we comprised analysis of contents of water, total ash, crude protein and crude fat. Highly significant difference ($p<0.01$) was found in the red meat for the contents of total ash, while the other parameters of chemical contents showed no statistically significant difference ($p>0.05$). Highly significant difference ($p<0.01$) was found in the white meat for the contents of water, total ash, crude protein; for crude fat is established statistically significant difference ($p<0.05$). Analysis of liver showed highly significant difference ($p<0.01$) for the contents of crude protein and crude fat, while highly significant difference ($p<0.01$) was established for the all chemical parameters in gizzard. Addition of sunflower oil to broilers' nutrition resulted with higher contents of total ash in the red and white meat, as well as of crude proteins and crude fats in the white meat, liver and gizzard of broilers.
Key words: *broiler meat, liver, gizzard, chemical contents, vegetable fats, animal fats*

INTRODUCTION

Poultry has a leading position in the demand for all types of meat in the world's most developed countries. This is the result of a series of factors, and the most important are: very short feeding duration, high concentration of live weight of poultry in the poultry house, the great reproductive power of breeding flocks, excellent feed conversion, nutritive value of poultry meat, relatively low selling price and the suitability through so-called " – fast food". Chicken is a significant diet animal product in human nutrition. It is characterized by high contents of undiminished protein and low fat content. The chemical composition of chicken's meat depends on various factors such as: age, rearing, nutrition and the body region, or a particular torso section. The meat on breast and leg meat with upper legs differ from one another in nutrients (Bašić, 2009). Given that there is a possibility to modify the content of desired ingredients in chicken meat, numerous studies are now turning in the direction of "designing" chemical composition of chicken meat. Using different sources of fat in the chickens' diet was the subject of study of many authors. The enrichment of chicken meat with the essential linoleic and linolenic acid is possible when as a food additive sunflower oil and soya oil are used instead of lard (Božić, 1997). Crespo and Esteve-Garcia (2001) suggest that feeding chickens with sunflower and linseed oil significantly reduce abdominal fat and cholesterol content in red meat ($p <0.01$) compared to consumption of chickens with lard and olive oil. Crespo and Esteve-Garcia (2001) analysed the contents of fats and proteins in the red and white meat of broilers in the experiment in which they supplemented to broiler's nutrition tallow, olive oil, sunflower oil and linseed oil (6% or 10%). Male broilers received the fats in their nutrition from 21. to 42. days of live, and female broiler's received the fats from 21. to 49. days of live. Results indicated that contents and type of fats did not significant influence on the weight and percent of drums an breast and contents of crude fat and crude protein in the mentioned tissues. Kirshgessner at all. 1993) have found the

enhancement contents of crude fat in the white meat of broilers which received in their nutrition higher percent of linoleic acid. Chickens fed with low-protein food (18% crude protein) supplemented with the oil enriched with 2% or 4% conjugated linoleic acid had low triglycerides of liver, a relatively high concentration of saturated fatty acids and relatively low concentration of monounsaturated fatty acids in lipids of liver and adipose tissue than chickens fed without the addition of conjugated linoleic acid. Chickens fed with low-protein food without the addition of conjugated linoleic acid had higher concentrations of triglycerides in the liver than chickens fed with high-protein food (23% crude protein) without the addition of conjugated linoleic acid (Aletor et al., 2003). When chickens were fed with 5 and 7.5% soybean oil and higher protein than the NRC recommendations (NRC x 1.1), Tabedian et al., (2005) there were recorded higher values of liver weight which mentioned authors attributed to increased activity of the liver for metabolism of these nutrients.

MATERIAL AND METHODS

The experiment was set up and implemented in the facilities for the production of chicken meat “Koka – Sana” from Sanski Most. Laboratory samples of chicken meat, liver and gizzard were performed at the Biotechnical Faculty, University of Bihać.
Day-old Cobb 500 broiler hybrid were placed in four separate boxes (treatments), and there were 60 broilers in each of them. All chickens were held on the floor in facilities fitted for broiler breeding. During the experiment, which lasted for 42 days, temperature, humidity and lighting were regularly controlled. Chicken breeding was split in two periods. From day one to day 15, chickens were bred with the initial mixture containing approximately 23% of proteins. From day sixteen to 42, they were bred with the final mixture containing approximately 20% of proteins, so the final mixtures were isoproteinic and isoenergetic. In chickens’ nutrition, there was increased content of fats by 3% (treatment I – lard, treatment II – soybean oil, treatment III – tallow, and treatment IV – sunflower oil). The chickens consumed food and water *ad libitum*. Having turned 42 days of life, chickens were marked with rings, for each treatment separately, and after 12 hours of fasting were killed at slaughterhouse facilities. After slaughter and meat packing processing of chickens’ carcasses, the carcasses and offal are chilled to a temperature of 0-4 ° C and then frozen at -18 °C until the moment of analysis, and on the day of analysis thawed to room temperature.
With analysis of chemical parameters of meat and offal we comprised analysis of contents of water, total ash, crude protein and crude fat according to to Official Methods of Analysis of AOAC International, AOAC International, Gaithersburg, USA (2000). The results obtained in the experiment were analyzed by ANOVA test and found differences were analyzed using Tukey’s test.

RESULTS AND DISCUSSION

In the Table 1 are shown the contents of nutrients in the broilers’ feeding. In the Table 2 are shown the chemical parameters of red and white meat. In the Table 3 are shown the chemical parameters of liver and gizzard.

Table 1. Contents of the mixtures used for feeding broilers from 0. to 15. days of life and from 16. to 42. days of live

Nutrients %	Experimental group							
	I/lard		II/soybean oil		III/tallow		IV/sunflower oil	
	0-15	15-16	0-15	15-16	0-15	15-16	0-15	15-16
Corn	53.5	58.5	53.5	58.5	53.5	58.5	53.5	58.5
Soybean shot	38.0	33	38.0	33	38.0	33	38.0	33
Sunflower shot	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Lard	3	3	-	-	-	-	-	-
Soybean oil	-	-	3	3	-	-	-	-
Tallow	-	-	-		3	3	-	-
Sunflower oil	-	-	-	-	-	-	3	3
Premix/s-starter, f-finisher	4 s	4 f	4s	4f	4s	4f	4s	4f

Table 2. Chemical parameters of red (R) and white (W) meat

Chemical parameters of red (R) and white (W) meat									F value
Water %	Lard		Soybean oil		Tallow		Sunflower oil		R meat - 1.254/NS W meat - 19.427**
	R	W	R	W	R	W	R	W	
$\overline{X}$	78.38	72.94	75.68	76.52	78.37	76.09	75.44	76.24	
SD	2.57	0.70	2.48	0.57	1.11	1.08	4.38	0.76	
CV	0.033	0.010	0.032	0.007	0.014	0.014	0.058	0.010	
Dry matter%	Lard		Soybean oil		Tallow		Sunflower oil		R meat - 1.888/NS W meat - 9.302**
	R	W	R	W	R	W	R	W	
$\overline{X}$	21.62	27.06	25.32	23.48	21.63	23.91	24.56	23.76	
SD	2.17	0.70	2.66	0.98	2.14	1.09	2.17	0.76	
CV	0.10	0.026	0.10	0.042	0.87	0.460	1.70	0.032	
Ash %	Lard		Soybean oil		Tallow		Sunflower oil		R meat - 5.571** W meat - 8.395**
	R	W	R	W	R	W	R	W	
$\overline{X}$	0.82	0.80	0.89	1.43	0.90	1.70	1.18	2.03	
SD	2.01	0.12	0.01	0.37	0.05	0.27	0.06	0.75	
CV	2.44	0.15	0.01	0.26	0.06	0.16	0.05	0.37	
Crude protein %	Lard		Soybean oil		Tallow		Sunflower oil		R meat- 0.674/NS W meat - 10.422**
	R	W	R	W	R	W	R	W	
$\overline{X}$	19.37	22.91	19.2	23.95	20.2	21.94	20.17	26.93	
SD	2.78	0.63	0.51	2.83	7.05	1.41	0.59	0.42	
CV	0.24	0.028	0.04	0.120	0.57	0.064	0.04	0.016	
Crude fat%	Lard		Soybean oil		Tallow		Sunflower oil		R meat - 0.248/NS W meat - 4.162*
	R	W	R	W	R	W	R	W	
$\overline{X}$	4.22	1.94	4.67	1.47	4.44	2.08	4.58	2.93	
SD	0.81	0.17	0.58	0.06	0.40	1.32	2.28	0.77	
CV	0.19	0.08	0.12	0.04	0.09	0.63	0.49	0.26	

F – values of Fisher test, $\overline{X}$ *- mean value, SD – standard deviation, CV – coefficient of variation, NS – Inside examined tretmans did not establish significant difference (p>0.05)*
*** Highly significant difference (p<0.01) between treatments, *significant difference (p<0,05) between treatments*

By using Tukey's test it was found highly significant difference (p <0.01) in the content of ash in the red meat of broilers between treatment I - lard and IV - sunflower oil.

By using Tukey's test it was found highly significant difference (p <0.01) in the content of water and dry matter in the white meat of broilers between treatments I and II, treatments I and III, and treatments I and IV. Highly significant difference (p <0.01) in the content of ash is established between treatments I and IV, and I and III. For the content of crude protein, highly significant difference is established for the treatments I and IV, and III and IV. Significant difference (p<0,05) is established for the content of crude fat between treatments II and IV.

Table 3. Chemical parameters of liver (L) and gizzard (G)

Chemical parameters of liver (L) and gizzard (G)									F value
Water %	Lard		Soybean oil		Tallow		Sunflower oil		L -0.441/NS G -16.290**
	L	G	L	G	L	G	L	G	
$\overline{X}$	71.04	75.20	81.30	81.27	78.79	80.61	78.85	79.97	
SD	2.52	3.50	5.41	1.04	1.66	1.00	2.58	0.39	
CV	0.03	0.05	0.07	0.01	0.02	0.01	0.03	0.005	
Dry matter%	Lard		Soybean oil		Tallow		Sunflower oil		L – 0.417/NS G -16.699**
	L	G	L	G	L	G	L	G	
$\overline{X}$	29.96	25.80	19.70	18.73	21.21	19.39	21.15	20.03	
SD	2.08	3.50	5.37	0.89	2.13	1.01	3.10	0.38	
CV	0.10	0.14	0.27	0.05	0.10	0.05	0.15	0.02	
Ash %	Lard		Soybean oil		Tallow		Sunflower oil		L-2.800/NS G-40.000**
	L	G	L	G	L	G	L	G	
$\overline{X}$	1.23	0.90	1.20	0.94	1.22	0.96	1.23	1.01	
SD	0.13	1.77	0.13	0.02	0.60	0.02	0.61	0.04	
CV	0.11	1.97	0.11	0.02	0.49	0.02	0.50	0.04	
Crude protein %	Lard		Soybean oil		Tallow		Sunflower oil		L-9.820** G-29.470**
	L	G	L	G	L	G	L	G	
$\overline{X}$	18.09	17.11	18.42	17.28	16.03	16.36	19.55	20.34	
SD	1.61	0.75	0.66	1.03	1.23	0.37	1.07	0.74	
CV	0.06	0.04	0.04	0.06	0.08	0.02	0.05	0.04	
Crude fat%	Lard		Soybean oil		Tallow		Sunflower oil		L-10.143** G-8.375**
	L	G	L	G	L	G	L	G	
$\overline{X}$	5.04	2.31	4.95	1.94	5.12	2.43	5.33	2.53	
SD	0.04	0.32	0.17	0.14	0.33	0.12	0.14	0.16	
CV	0.01	0.14	0.03	0.07	0.06	0.04	0.02	0.06	

F – values of Fisher test, $\overline{X}$ - mean value, SD – standard deviation, CV – coefficient of variation, NS – Inside examined tretmans did not establish significant difference (p>0.05)
*** Highly significant difference (p<0.01) between treatments*

We were found highly significant difference (p <0.01) in the content of crude protein between treatments III and IV, and crude fat between treatments I and IV and II and IV in the liver of broilers. Highly significant difference (p <0.01) was established in the content of water and dry matter between treatments I and II, I and III, I and IV in the gizzard; for the content of ash we were found highly significant difference (p <0.01) between treatments I and II, I and IV; for the content of crude protein we were found highly significant difference (p <0.01) between treatments I and IV, III and IV, III and IV; for the content of ash we were found highly significant difference (p <0.01) between treatments II and III, II and IV in the gizzard.

CONCLUSIONS

Addition of animal fat and vegetable oil to broiler's feeding were significantly influenced on the contents of ash of red meat of broiler chickens, while the other parameters of chemical contents did not influence, but for the chemical parameters of white meat, addition of animal fat and vegetable oil to broiler's feeding were significantly influenced on the contents of water, dry matter, ash, crude protein and crude fats. Addition of sunflower oil to broilers' nutrition resulted with higher contents of total ash in the red and white meat, as well as of crude proteins and crude fats in the white meat of broilers. For the chemical parameters of liver and gizzard, addition of animal fat and vegetable oil to broiler's feeding were significantly influenced on the contents of crude protein and crude ash.

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EFFECT OF DIFFERENT CONCENTRATION OF LINSEED OIL IN THE DIETS ON CONTENT OF ESSENTIAL FATTY ACIDS OF COMMON CARP, CYPRINUS CARPIO, L

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ABSTRACT: Common carp fingerlings were fed five diets in triplicate containing 0, 2, 3, 4 and 5% linseed oil for 75 days to determine effect of different concentration of LO on chemical and fatty acid composition of fish. Fish, average initial weight 200± 4 g, were stocked in 15 cages. Fish fed the 3, 4 and 5% LO diets had significantly better growth parameters compared to fish fed the 0 or 2% LO diets. Lipid content in muscle increased from 1.25% in the 0% LO, to 1.46%; 1.56 %; 1.94% and 2.37% in the 2, 3, 4 and 5% LO treatments, respectively. Fatty acid profiles in muscle tissue reflected the diet concentrations with significant increases ($p < 0,01$) in 18:3n-3 in fish fed the 2, 3, 4 and 5% LO diets. Other fatty acids showing significant ($p < 0,01$) muscle concentration increases in fish fed the LO diets were: C20:3n-3, C20:5n-3, C22:5n-3, C22:6n-3, as well as polyunsaturated fatty acids (PUFA), total n−3 fatty acids, and the n-3/n-6 ratio. Fatty acids which showed significant decreases ($p<0,01$) were saturated fatty acids (SFA). Fish fed the 5% LO diets had twice much n−3 fatty acids (7.05%) than those without LO (2.65%). N-3/n6 ranged from 0.14 in fish fed without LO to 0.33 in fish fed 5%LO. Inclusion of 5% LO in diets showed the most favourable effects on content of essential fatty acids in tissue of carp as well as on other tested parameters.
Key words: *common carp, cages, chemical composition, fatty acid, linseed oil*

INTRODUCTION

Regarding the fact that the intake of linoleic acid relative to α-linolenic acid in modern human diets is very high, dietary linseed oil can prove to be a key feed ingredient in the establishment of common carp meat as a food useful for decreasing the risk of cardiovascular diseases in humans (Zelenka et al., 2003). Linseed oil is acceptable from the viewpoint of human nutrition based on the innate ability of cyprinids to convert dietary α-linolenic acid to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Buzzi et al., 1996; Bell et al., 2001). The tested vegetable oil was selected because of its availability and price, which make it suitable for fish feed industry. It has long been known that, providing their essential fatty acids requirements are met, many freshwater fish can be successfully reared on diets containing vegetable or terrestrial animal oils (Sargent et al., 2002).
Feed composition becomes very important in intensive fish farming, apart from hydroecology, genetics and pathology, because natural feed loses its significance in cage fish farming conditions (Bogut et al., 2002).
The effect of linseed oil on growth and meat characteristics of young common carp was carried out at a fish farm in Grabovo, Croatia, in duration of 75 days, started on April and finished on June.

MATERIAL AND METHODS

The cage platform with 15 cages was set up in a 650-m2 fishpond. Water was added after the fishpond had been disinfected with 160 kg of Ca (HCO3)2. Five experimental fish groups (C, L2, L3, L4 and L5) were included in the trial. Each dietary treatment was tested in three replicates. At the beginning of the experiment each cage was colonized with 60 1-year-old common carp; mean body mass per cage ranged from 198 to 206g/ind. Prior to the experiment the fish were bred under fishpond conditions in a monoculture and fed with pelleted feed.

Feed mixtures

Composition of the fish feed is presented in Table 1. Pellet diameter was 4.5 mm. Fish in the first three cages received feed without addition of linseed oil (control group C, cages 1, 2 and 3), while the other cages received the same quantity of feed with different quantities of linseed oil: 2.0% in L2 (cages 4, 5 and 6); 3.0% in L3 (cages 7, 8 and 9); 4.0% in L4 (cages 10,11 and 12) and 5.0% in L5 (cages 13, 14 and 15) (Table 1).
Daily feed amount was given in three rates at 8.00, 13.00, and 17.00 h; the schedule was determined according to feeding tables on the basis of water temperature, dissolved oxygen concentration and other physical and chemical water parameters. The fishes were not fed during the stocking day or during control fishing.

Analyses

Upon experiment completion, the body mass of all fishes was determined. Three fish were taken from each cage for further chemical analysis. The fishes were chilled and the bones and skin separated from the muscle tissue. Chemical composition of fish muscle tissue and experimental feed mixtures was determined using standard SRPS ISO methods as described by Trbović et al. (2009). Protein content was determined by Kjeldahl method (Kjeltec Auto 1030 Analyzer, Tecator, Sweeden). Water content was determined by drying at 103±2ºC to constant weight. For determination of total fat, the samples were hydrolyzed with 4M hydrochloric acid and extracted with petroleum ether by Soxhlet apparatus. Ash content was determined by combustion at 550±25ºC. Fatty acids determination was performed according to Spirić et al. (2009) by capillary gas chromatography. Group effect was determined using one-way ANOVA (Statistica 10.0, StatSoft Inc.). Inter-group differences were attained by the Tukey HSD test at $p \leq 0.01$. The results were presented as means±SE.

Growth performance

Growth-performance indicators [specific growth rate (SGR, % weight day-1), feed conversion ratio (FCR), weight gain (WG, %) and survival rate (SR, %)] were measured using following formulas:

SGR = 100 (Ln (mean final body weight)-Ln (mean initial body weight))/time (days);
FCR = dry feed intake (g)/wet weight gain (g);
SR (%) = (Final fish number/initial fish number)*100;
WG = Final body weight (g) - initial body weight (g) (g fish^{-1}**).**

RESULTS AND DISCUSSION

Chemical analysis results in the meat of common carp that had been fed with different linseed oil quantities are presented in Table 3. The increase in linseed oil content is accompanied by a slight decrease in fish meat water content, ranging from 79.16% in C to 77.84% in L5. On the contrary, the fat and protein content increases with higher linseed oil content, ranging from 1.25% in C to 2.37% in L5 and from 17.13% in C to 18.87% in L5, respectively. The ash content was the same in all analysed groups.

The addition of linseed oil to fish feed influenced the fatty acid composition of the fish meat fat (Table 4). Increased linseed oil content in feed is accompanied by a lower saturated and monounsaturated fatty acid content in fish meat. At the same time, detected was an increase in polyunsaturated, especially n-3 fatty acids, as well as a pronounced increase in the ratio of n-3 versus n-6 fatty acids (Table 4).

Table 1. Composition and proximate analysis of the extruded formulated diet

Ingredients (%)	C	L2	L3	L4	L5
Soybean meal	50	50	50	50	50
Sunflower meal	18	18	18	18	18
Brewery yeast	5	5	5	5	5
Linseed oil	0	2	3	4	5
Wheat flour	10,6	10,6	10,6	10,6	10,6
Corn	12	10	9	8	7
Methionin	0,1	0,1	0,1	0,1	0,1
Lysine L	0,3	0,3	0,3	0,3	0,3
Vitamin mix1	2	2	2	2	2
Mineral mix2	2	2	2	2	2
Chemical analysis (%)					
Dry matter	89,91	90,15	90,27	90,38	90,5
Crude protein	32,49	32,33	32,25	32,17	32,09
Crude fat	1,56	3,46	4,4	5,36	6,31
Crude ash)	4,49	4,46	4,45	4,44	4,43
NFE[3]	61,45	59,74	58,88	58,03	57,17

[1]Vitamin mix(mg kg-1 of diet): vitamin B1, 15; vitamin B2, 10; vitamin B6, 20; vitamin B12, 0,15; vitamin K3, 15; inositol, 250; Ca-pantothenic acid, 80; nicotinic acid, 100; folic acid, 1; vitamin H (biotin), 1; vitamin E, 140; vitamin C, 500; vitamin A, 20 000 IU; vitamin D3, 6 000 IU; choline chloride, 1 800, and cellulose was used as a carrier.

[2]Mineral mix (mg kg-1 of diet): Cu 20, Fe 40, Mn 30, Se 0.4, Zn 125 , and cellulose was used as a carrier

[3]NFE, nitrogen-free extract, g.kg-1 DM = 100 – (CP + CF + CA)

Growth performance was significantly affected by addition of linseed oil in fish diets (Table 2). It should be noted that the growth rate of carp fed diets supplemented with linseed oil in this study was significantly faster than that of fish fed diets without supplementation of linseed oil. Concentration of added oil also showed significant influence on the growth performance. This could indicate that linseed oil has a favourable impact on the growth of common carp. Similar results were reported by Bogut et al. (2002) for catfish and (Zakes et al., 2010) for tench.

Table 2. Growth performance of experimental fish

Variable[2]	**C**	**L2**	**L3**	**L4**	**L5**
Initial number of fish	180	180	180	180	180
IBW (g)	201.67±1.53	203.67±2.52	201±1	201±2.65	201±1
FBW (g)	484.3 ± 4.04^{a}	492.33 ± 4.93^{ab}	502 ± 2^{ab}	508 ± 2^{b}	519.67 ± 2.52^{c}
Final number of fish	141	147	150	147	153
Survival rate (%) SR	78.33	81.67	83.33	81.67	85
WG ($gfish^{-1}$)	282.67 ± 2.51^{a}	288.67 ± 2.42^{ab}	301 ± 1.73^{ab}	307 ± 3.61^{bc}	318.67 ± 3.5^{c}
SGR ($\%\cdot day^{-1}$)	$1,17\pm1.29^{a}$	1.18 ± 0.03^{ab}	1.22 ± 0.01^{ab}	1.24 ± 0.02^{bc}	1.27 ± 0.01^{c}
FCR ($g\cdot g^{-1}$)	2.16 ± 0.04^{a}	1.93 ± 0.03^{b}	1.76 ± 0.01^{c}	$1,79\pm0.03^{c}$	$1,59\pm0.02^{d}$

Data are means ± SE (n = 3). Values within the same row with different letter superscript differ at p<0.01, IBW, initial body weight; FBW, final body weight; SR, survival rate; SGR, specific growth rate; FCR, feed conversion ratio; WG, weight gain;

Table 3. Chemical composition of experimental fish

Parameters (%)	C	L2	L3	L4	L5
Moisture content (%)	79,16±0,07[a]	78,87±0,16[ab]	78,61±0,07[b]	78,53±0,03[b]	77,84±0,15[c]
Protein content (%)	17,13±0,08[a]	17,99±0,14[b]	18,24±0,02[b]	18,62±0,03[c]	18,87±0,1[c]
Fat content (%)	1,25±0,12[a]	1,46±0,23[a]	1,56±0,1[a]	1,94±0,05[b]	2,37±0,28[c]
Ash content (%)	0,99±0,03	0,99±0,03	0,98±0,03	0,96±0,01	1,01±0,05

Values are means ± SD (n = 3); Values in the same row with different letter notation statistically significantly differ at p < 0.01/

Table 4. Fatty acid composition of experimental fish

	C	**L2**	**L3**	**L4**	**L5**
Fatty acid (%)					
C14:0	0,93±0,07	0,92±0,1	0,95±0,02	0,91±0,02	0,94±0,06
C15:0	0,16±0,03	0,17±0,01	0,17±0,00	0,17±0,01	1,63±0,01
C16:0	21,29±3,58	22,25±2,03	20,07±0,16	20,36±0,49	19,12±0,6
C16:1	5,33±0,58	4,96±0,4	5,21±0,51	5,29±0,75	4,85±0,51
C17:0	0,27±0,04	0,29±0,03	0,28±0,02	0,28±0,04	0,26±0,01
C18:0	4,67±1,66	5,16±0,83	5,01±0,15	4,52±0,37	4,54±0,36
C18:1cis-9	38,29± 2,67[ab]	35,27± 1,94[ab]	39,34± 2,81[ab]	36,23± 0,73[a]	39,20± 0,78[b]
C18:1cis-11	5,12± 0,17a	4,73± 0,24a	1,65± 2,86ab	4,43± 0,22a	0,00±0,00b
C18:2 ω-6	15,42± 3,32	14,93± 0,44	15,21± 0,72	15,46± 1,00	17,72±1,34
C18:3 ω-6	0,15±0,02	0,19±0,0,06	0,13±0,01	0,15±0,04	0,19±0,09
C18:3 ω-3	0,56± 0,29[a]	1,12± 0,33[a]	1,73± 0,08[b]	1,86±0,05[b]	2,75± 0,19[c]
C20:0	0,21±0,03[ab]	0,27±0,05[ab]	0,26±0,01[ab]	0,25±0,02[a]	0,20±0,01[b]
C20:1	2,54± 0,24	2,38± 0,15	2,44± 0,25	2,31± 0,37	2,31± 0,37
C20:2	0,46± 0,05	0,45±0 0,07	0,49± 0,02	0,45 0,09	0,44±0,09
C20:3 ω-6	0,84±0,16	0,86±0,14	0,70±0,03	0,69±0,07	0,62±0,06
C20:3 ω-3	0,21±0,04[a]	0,4±0,07[b]	0,5±0,01[b]	0,62±0,07[bc]	0,73±0,04[c]
C20:4 ω-6	1,70±0,48	2,57±0,07	2,65±0,06	2,76±0,04	2,8±0,01
C20:5 ω-3	0,18±0,02[a]	0,22±0,01[a]	0,25±0,01[b]	0,28±0,01[b]	0,31±0,02[c]
C22:5 ω-3	0,16±0,02[a]	0,21±0,02[a]	0,27±0,02[b]	0,30±0,01[bc]	0,36±0,03[c]
C22:6 ω-3	1,54±0,26[a]	2,67±0,1[b]	2,68±0,02[c]	2,79±0,02[d]	2,9±0,07[e]
SFA	27,52±5,25[abc]	29,07±2,86[abc]	26,75±0,33[b]	26,5±0,53[abc]	25,22±0,3[c]
MUFA	51,28±3,42	47,34±2,49	48,64±0,4	48,28±0,96	46,37±0,69
PUFA	21,21±3,42[ab]	23,63±0,35[a]	24,59±0,86[a]	25,36±1,02[b]	28,83±1,26[b]
∑ ω -6	18,56±3,05	19,01±0,33	19,17±0,81	19,51±0,92	21,78±1,44
∑ ω -3	2,65±0,4[a]	4,62±0,34[b]	5,43±0,07[c]	5,85±0,12[d]	7,05±0,18[e]
ω-3/ω-6	0,14±0,01[a]	0,24±0,02[b]	0,28±0,01[b]	0,29±0,01[b]	0,33±0,02[b]

Values are means ± SD (n = 8); Values in the same row with different letter notation statistically significantly differ at p < 0.01. SFA-saturated fatty acids; MUFA-monounsaturated fatty acids; PUFA-polyunsaturated fatty acids from the n-3 (n-3 PUFA) and n-6 (n-6 PUFA) families

The fatty acids profiles of fish body is strictly linked to their dietary content (Bell, 1998; Zakes et al., 2010; Ćirković et al., 2011) and this means that fatty acid profiles of tissues can be modified by altering the types of fats and oils used in feed (Francis et al., 2006; Sargent et al., 2002; Turchini et al., 2003).

CONCLUSIONS

The addition of 2-5 % linseed oil to the feed of common carp has multiple beneficial consequences on fish breeding. It improves fish growth indicators and fish meat quality (increases the protein content and polyunsaturated fatty acids proportion).

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TP 31011 “Uticaj kvaliteta komponenata u ishrani ciprinida na kvalitet mesa, gubitke i ekonomičnost proizvodnje (The

influence of the quality of the components of food for cyprinid fish species on the quality of meat, losses and the profitability of production)", financed by the Ministry of Science and Technological Development, Republic of Serbia.

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EFFECTS OF *ARTEMISIA ABSINTHIUM* ON COCCIDIA INFECTIONS IN CHICKENS

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ABSTRACT: This report was designed to compare the efficacy of Artemisia absinthium L. powder and amprolium on the basis of weight gain feed conversion ratio, oocyst count and mortality rate. A total of 90, day-old broiler chicks of both sexes were randomly divided into six groups (A to F). From first day onward, ration was supplemented with 1; 2 and 3% Artemisia absinthium L. powder in groups B and D, respectively, group A received amprolium: 60 ppm in drinking water, while groups E and F kept as infected un-medicated controls. First five groups were infected with Eimeria tenella sporulated ooccysts: 30.000/chick at the age of 20th. Maximum coccidiostatic effect was observed with Artemisia absinthium L. (3%) showing mild bloody diarrhea as compared to other infected groups receiving A. absinthium L. containing rations. Anticoccidial effect of used herb and coccidiostatic- amprolium, suggests that further studies should be carried out to determine the possible maximum safe levels with least toxic effects to be used as coccidiostat.
Key words: *Artemisia absinthium L., coccidiosis, amprolium, broilers*

INTRODUCTION

The genus Eimeria comprises a population of obligate intracellular protozoan species with a complex life-cycle including both asexual and sexual stages of development. Coccidiosis is the consequence of Eimeria infection of the intestine of the permissive host. This condition markedly reduces growth and feed utilization in poultry and livestock. Due to intensive breeding of poultry, coccidiosis is a lingering economical problem for poultry industries worldwide. Many anticoccidial drugs have been developed and introduced in the poultry industry all over the world. The increasing resistance of avian coccidia (protozoa) to anticoccidial drugs currently used by the poultry industry has stimulated the search for new methods of control. Therefore, there is need to find out the safe alternatives for the control of avian coccidiosis. In this context, a number of plants and herbal products have been found to be effective for a broad range of parasites such as protozoa, arthropods and helminates (Akhtar and Rifaat, 1985; Jiang et al., 1985; Klayman, 1985; Cooke et al., 1987; Quan, 1990). As part of this effort we have investigated *Artemisia absinthium* as a potential source of compounds with anticoccidial activity.

The genus Artemisia belongs to the family Compositae (*Asteraceae)* and has over 300 species spread worldwide. Plants from this family are a rich source of sesquiterpene lactones, a class of natural compounds with several proved medicinal effects, including anthelmintic effect. In the past 8-10 years, new medicinal benefits were reported for several *Artemisia* species (spp.) due to the anti-parasitic effects of some artemisinin-based compounds and the high antioxidant capacity of crude extracts of some plants of this genus. Besides artemisinin, *Artemisia* spp. are rich sources of sesquiterpene lactones and antioxidant compounds (flavonoids, phenolic acids, etc.) with potential benefits to human and animal health (Orav et al., 2006).

Information regarding anticoccidial activity of herbs and homeopathic products is very limited. Therefore, the present study was carried out to evaluate the anticoccidial effect of *Artemisia absinthium* in comparison with amprolium, a standard anticoccidial in broilers.

MATERIAL AND METHODS

Birds

The experimental protocol was approved by the local Ethics Committee; the principles of animal protection were strictly followed. Experiments under *in vivo* conditions were performed on ninethy broilers of the heavy line Arbor Acres, of both sexes. One –day-old broilers, randomly selected, were divided into six groups (A to F), each numbering 15 individuals. Bird fed a standard basal diet. All birds had free access to water and feed. Temperature and lighting regimens were in accordance with the recommenditation of the breeder. The initial room temperature 32-33º C was reduced weekly by 1ºC to a final temperature of 28ºC.

Parasite and dose

Coccidial oocysts of *Eimeria tenella* spp. were obtained from the guts of infected chicks and propagated in broiler chicks by giving oral infection. The oocysts were preserved in 2,5% potassium dichromate solution to induce sporulation and kept in a refrigerator (2-5ºC) until use. Each bird was challenged with 30.000 oocysts/chicken of *E. tenella* at the age of 20th day.

Experimental groups

There were six experimental groups and each was having 15 chicks. Different groups of chicks were assigned to various rations with different supplementations from day 1 till end of experiment. The infection was given on 20th day-of age to all the groups except group F, which served as uninfected un-medicated group. The medication with herbal powder and standard coccidiostatic was started according to the following schedule.

Group A: Amprolium (Kokciprol): 60 mg per 1 dm^3 of drinking water;
Group B: *Artemisia absinthium* crude powder: 1%;
Group C: *Artemisia absinthium* crude powder: 2%;
Group D: *Artemisia absinthium* crude powder: 3%;
Group E: Infected unmedicated control;
Group F: Uninfected unmedicated control.

Evaluation parameters

Efficacy of *Artemisia absinthium* powder and amprolium was evaluated on the basis of body weight gain, feed consuption, feed conversion rates, bloody diarrhea and oocyst counts. The body weight gain and feed conversion ratio was determined on weekly basis up to the end of experiment (six weeks). Bloody diarrhea was investigated from 4th to 6th day after the challenge. The extent of bloody diarrheal score was assigned one of the four degrees, from 0(-) to 3(+++). Zero was the normal status, whereas 1,2 and 3 corresponded to 33, 33-66, 66-99% blood in total feces, respectively. OPG (oocysts per gram of faeces) were counted from 6 to 13 days after infection with *E. tenella.*

Statistical analysis

The data of body weight gain, feed conversion rates and feed intake were analyzed statistically by repeated measures analysis of variance (Steel and Torrie, 1982).

RESULTS AND DISCUSSION

The mean values of body weight gain, feed consuption and feed conversion ratio in various groups at different weeks after the treatment are shown in table 1. During first three weeks, before inoculation of infection, the body weight gain, feed consuption and conversion ratio were not significantly different among the groups. At the end of experiment, the body weight gain in the groups treated with amprolium and 3% *Artemisia absinthium* powder (2316g and

2329g, respectively) were significantly higher than that of infected group (1991g). The body weight gain in the groups treated with rations supplemented with 1% and 2% *Artemisia absinthium* powder (2024g and 2059g, respectively) were relatively, but not significantly higher than that of infected group.

Table 1. Broiler production performance (0-6 weeks)

Groups	Average feed intake/bird (g)	Average wt gained/bird (g)	Feed conversion ratio
Amprolium (A)	4606[b]	2316[b]	2,05[a]
1% *Artemisia absinthium* powder (B)	4133[a]	2024[a]	2,26[b]
2% *Artemisia absinthium* powder (C)	4218[a]	2059[a]	2,24[b]
3% *Artemisia absinthium* powder (D)	4613[b]	2329[b]	2,01[a]
Infected control (E)	4121[a]	1991[a]	2,30[b]
Uninfected control (F)	4618[b]	2334[b]	1,98[a]

Means in each row lacking common superscript differ significantly (P<0.05)

The body weight gain, feed consuption and feed conversion rate of all the groups were investigated from 1st to 6th week. The body weight gain and feed consuption of all the infected groups were lower than that of uninfected control group. But, the body weight gain and feed consuption of groups supplemented with the rations containing amprolium and 3% turmeric powder were significantly greater than that of all other infected groups. Also, the body weight gain and feed consuption of groups supplemented with the rations containing 1% and 2% *Artemisia absinthium* powder were greater than that of infected group but that difference was not significant.

Bloody diarrhea of almost all experimental groups, with the exception of the uninfected control group, was observed from the 4th to 6th day after challenge with *E. tenella.* In the groups treated with rations supplemented with amprolium, and 2% and 3% *Artemisia absinthium* powder, the extent of bloody diarrhea was milder than that observed in various groups at different weeks after the treatment are shown in table 2*.*

Table 2. Bloods diarrhea of chickens treated with *Artemisia absinthium* powder and challenged with *Eimeria tenella.*

Groups	Blood in feces (days after infection)				
	3	4	5	6	7
Amprolium (A)	-	+	+	-	-
1% *Artemisia absinthium* powder (B)	-	+	++	+	-
2% *Artemisia absinthium* powder (C)	-	+	++	-	-
3% *Artemisia absinthium* powder (D)	-	+	+	-	-
Infected control (E)	-	+	+++	+	-
Uninfected control (F)	-	-	-	-	-

After challenge with *E. tenella*, the bloody diarrhea and excreted oocysts of feces were investigated during two weeks. Bloody diarrhea of all the experimental groups, except the uninfected control group were seen during 4-6 days after infection with *E. tenella.* But the extent of bloody diarrhea in the groups treated with ration supplemented with amprolium and 3% *Artemisia absinthium* powder was milder than that of other groups. Excreted oocysts in the groups treated with ration supplemented with 1% and 2% *Artemisia absinthium* powder were relatively lower than that of the infected control group. In the groups treated with ration supplemented with 3% *Artemisia absinthium* powder and amprolium, the peak excretion of oocysts was delayed about 1 or 2 days relative to the control infected group (table 3)*.*

Table 3. Oocyst excretions and mortality of chickens treated with *Artemisia absinthium* powder and challenged with *Eimeria tenella*

Groups	Average oocyst count g^{-1}		Mortality rate %
	Before treatmen	After treatmen	
Amprolium (A)	6500	1956	4(15)
1% *Artemisia absinthium* powder (B)	5500	19640	6(15)
2% *Artemisia absinthium* powder (C)	4300	15090	5(15)
3% *Artemisia absinthium* powder (D)	6000	2004	5(15)
Infected control (E)	4300	52200	6(15)
Uninfected control (F)	-	-	-

The oocysts out put and mortality rate were lower in all the treated groups as compared to infected un-medicated control group. However, among treated groups the birds treated with 3% *Artemisia absinthium* powder and amprolium showed better results in terms of oocyst count per gram of faeces and mortality rate as compared to those treated with 1% and 2% *Artemisia absinthium* powder. The counts were zero in uninfected groups.
The parasite was not completely suppressed by any of the treatment. The anticoccidial did not work in this case. The reason for this observation could be the resistance caused by anticoccidials (Butaye et al., 2000). Sluis (1998) also reported that severity of test exposure is important as resistant to anticoccidials was often effective with mild coccidiosis but ineffective at moderate or severe exposure. It has been reported that the diets supplemented with the different concentrations of the *Artemisia absinthium* were suppresive towards the development of coccidiosis in chickens. Furthermore, these diets were shown to increase the body weight gain, improve lesion scores and decrease oocysts out put.

CONCLUSIONS

It was concluded that the plant evaluated (*Artemisia absinthium)* in the study have anticoccidial activity.
Maximum coccidiostatic effect was observed with *Artemisia absinthium* (3%) showing mild bloody diarrhea as compared to other infected groups receiving *Artemisia absinthium* containing rations. Also, the weight gain in the group treated with 3% *Artemisia absinthium* was significantly higher ($p<0.05$) than that of infected control group.
It could be concluded that investigated plant (*Artemisia absinthium)* could be a potential source of protection against coccidiosis.

ACKNOWLEDGEMENTS

This paper is a result of the research within the projects III046012 and TR31031 financed by the Ministry of Education and Science, Republic of Serbia.

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INFLUENCE OF GASTROINTESTINAL HELMINTHS TO GOAT HEALTH AND PRODUCTION

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ABSTRACT: Goat's gastrointestinal helminths infection was of great importance to health status of goats and performances. This was parasitic infection caused by helminths from genus *Ostertagia, Trichostrongylus, Nematodirus, Chabertia, Haemonchus, Marshallagia, Skrjabinema* and *Oesophagostomum*. Goars infection had high prevalence, moderate morbidity and low mortality rate. Animals had abdominal pain, anemia, lost of appetite, dehydration, tenisms, weakens and lost of weight. Clinical signs are greenish or yellow diarrhea with smell, and some time is presented a blood. Young animals are depressive and inactive. Consequence is significant increase of kid accrescense, its weakens and less growth.
Key words: *gastrointestinal helminths, goats*

INTRODUCTION

During last decade goat production has started to play an important role in Serbia in providing animal protein for diet, especially for people living in villages. Goats reared in the area are of milk aptitude, but also constitute a meat supply for the consumers.
Goats gastrointestinal helminths infection was infection caused by helminths from genus *Ostertagia*, *Trichostrongylus*, *Nematodirus*, *Chabertia, Haemonchus, Marshallagia*, *Skrjabinema* and *Oesophagostomum* (Georgievski, 1989, Mishra, 1991). Goars infection had high prevalence, moderate morbidity and low mortality rate. Symptoms of acute greenish or yellow diarrhea with smell, and some time is presented a blood, and weight loss (Smith, 1990, Ilić, 1991).
No systematic studies had been previously made to determine the endoparasitic fauna of goats in Serbia. Examination of goat parasitoses has been sporadically performed and there are only a limited number of publications about it (Vujić and Bošković, 1981, Ilić, 1990, Vujić et al. 1991, Ilić et al. 1991, Pavlović et al. 1995).
In the present study we performed an extensive examination of parasitic fauna of goats at various parts of Serbia during a two year period (2010-2011) obtained during realization of project TR 31053.

MATERIAL AND METHODS

Our examination was performed in period 2010-2011. We examined 331 goat and sheep flock (usualy breed together) from 19 villages in the area of Belgrade. Total of 1450 faeces samples were examined using routine coprological methods (Zuriilski and Rusev, 1990). A total of 67 goats were slaughtered for post-mortem examination. Total differential worm counts were done on all the gastrointestinal tract and lungs using the technique described by Pavlović and Anđelić-Buzadžić (2010). Determination of adult and eggs of parasites were done by keys given by Euzeby (1981) and Pavlović and Anđelić-Buzadžić (2011).

RESULTS AND DISCUSSION

The faecal samples were obtained from a different source all together as they were collected from flocks in the field, and the results support the other findings. These counts were also of

value in providing some information on the peripartuirent egg rise. The number of guts and lungs examined in this survey thought small in number, but in combination with results of coprological examination, samples appeared to represent the population adequately.
In period 2009-2010 we found next helminth species: *Ostertagia circumcincta* (95,23%), *O.trifurcata* (91,53%), *Trichostrongylus axei* (100%), *T.colubriformis* (89,57%), *Nematodirus spathiger* (100%), *N. filicolis* (43,31%), *Haemonchus contortus* (88,95%), *Marshallagia marshalli* (23,77%), *Skrjabinema caprae* (13,28%), *Chabertia ovina* (64,14%) and *Oesophagostomum venulosum* (28,39%).
Most prevalent species of nematode were Trichostrongylus and Nematodirus species. Although most of the gastro-intestinal species appear to follow this general pattern of seasonal distribution, some variations in intensity and duration of these characteristics with different worm species occurred. Thus with *Trichostrongylus* and *Nematodirus* species infection at mature sheep the spring peak was more pronounced that the autumn infection. When we compared our results to the similar survey at mountain area of Serbia that have been done at Šara Mountain (Vujić and Bošković, 1981, Vujićet et al., 1991, Pavlović et al., 1995), and at East Serbia (Ilić,1990, Ilić et al.1991, Pavlović et al.,1991, 2003), we were concluded that dominant endoparasite species were *Trichostrongylus spp*. and *Nematodirus spp.* Same parasite species were diagnosed in other Balkan countries like Macedonia or Bulgaria (Georgievski,1989, Zurliiski and Rusev,1990).
Generally speaking the occured parasites represent a global problem. Way of breeding usualy at shepeng had prerequisite to a lot of infections including parasitoses. Pasture breeding make possible contact within sheep and eggs, larval stages and intermediate host of parasites. Those induce that there are no one goats without parasites. The countries of Magreb, Middle East and Northern Africa have also permanent problem with parasitic infections and losses ensued by them.
From these reasons both infection can be prevented by adding anthelmintic drugs to the goat's diet. With careful management and sound preventive measures, the losses associated with this disease can be reduced to minimal levels.

CONCLUSIONS

Goat’s gastrointestinal helminths infection was of great importance to health status of goats and performances. This was parasitic infection caused by helminths from genus *Ostertagia*, *Trichostrongylus*, *Nematodirus*, *Chabertia, Haemonchus, Marshallagia*, *Skrjabinema* and *Oesophagostomum*. Goars infection had high prevalence, moderate morbidity and low mortality rate. Consequence is significant increase of goats accrescense, its weakens and less growth
The best preventive measure a goat producer can take is to use a feed with anthelmintic drugs added. With careful management and sound preventive measures, the losses associated with this disease can be reduced to minimal levels.

ACKNOWLEDGEMENTS

Our work are part of results from project TR 31053, "Implementation of new biotechnological solutions in breeding of cattle, sheep and goats for the purpose of obtaining biologically valuable and safe food”, funded by the Ministry of Education and Science of the Republic of Serbia.

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PREDICTION OF BIOAVAILABLE ENERGY OF FEED FOR POULTRY BY ESTIMATION OF ORGANIC MATTER DIGESTIBILITY

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ABSTRACT: One of the most important parameters of feed quality is energy. Not all energy of feed (gross energy) will be utilized by the animal, but only its bio-available portion, i.e. metabolisable energy (ME). The accepted method for direct determination of ME of feeds is by *in vivo* trials. These are often time-consuming and expensive why there has been a need for *in vitro* methods for predicting the *in vivo* ME values of feeds. The aim of this study was to develop linear equations to predict the *in vivo* true metabolisable energy (TME) of poultry feeds based on digestibility of organic matter as determined by an *in vitro* method. Sixteen diets and fifty seven samples of feedstufs for poultry were used. *In vivo* determination of TME was based on digestibility of nutrients, by use of the adult rooster assay. Same feed samples were analysed by an enzyme incubation based laboratory procedure for estimating the enzymatic digestible organic matter (EDOM). Obtained EDOM values were regressed against the *in vivo* TME results, which generated the prediction equation TME = 0.191 x EDOM, with R^2 = 0.704 (RSD = 0.127). When other predictors were included, prediction was improved and the following equations and R^2 have been generated: TME = 0.177 x EDOM + 0.168 x Fat, with R^2 = 0.766 (RSD = 0.113) and TME = 0.191 x EDOM + 0.209 x Fat – 0.044 x Protein, with R^2 = 0.830 (RSD = 0.098). The conclusion was that *in vivo* ME of feeds for poultry can be sucessfully predicted by use of organic matter digestibility.
Key words: *Metabolisable energy, prediction, poultry, feeds, organic matter digestibility*

INTRODUCTION

One of the most important parameters of feed quality is its energy. Not all energy of the feed (gross energy) will be utilized by the animal, but only a bio-available portion called metabolisable energy (ME). This parameter serves as an accurate indicator of feed quality and can be reliably used for feed quality control. Metabolisable energy is directly proportional to digestibility of nutrients, as it directly affects their availability and absorption (Čolović et al., 2011).

The accepted method for direct determination of ME of feeds is by *in vivo* trials. These are often expensive and time-consuming. *In vitro* methods used for predicting ME are attractive because of rapidity and low cost (Farrel, 1999) and can be estimated directly from parameters accessible in the feeds (Noblet and Perez, 1993). Therefore, there has always been a need for reliable laboratory methods and related equations for prediction of the *in vivo* ME values of feeds, in order to implement an adequate system of quality control (Alvarenga et al., 2011). There has been a considerable and continuous interest to develop equations for rapid and accurate prediction of ME (Perai et al., 2010; Robbins and Firman, 2005; Zhang et al., 1994).

The aim of this study was to develop equations, using linear model, for predicting the *in vivo* ME of poultry feeds from the digestibility of organic matter, as determined by an *in vitro* method not used for poultry feeds.

MATERIALS AND METHODS

A total of 57 feedstuff samples and 16 commercial complete diets for poultry was used in this study.

Proximate analysis
Dry matter (DM), crude protein (Protein), crude fibre (CF), crude fat (Fat) and Ash were determined according to AOAC official methods (2000).

***In vitro* determination of enzyme digestible organic matter (EDOM)**
The three-step procedure of Boisen and Fernadez (1997) was modified to 2-step thus using incubation of feed sample with pepsin for 75 min, followed by incubation with pancreatin for 18 h. Solubilised protein was precipitated with sulphosalicylic acid. Insolubilised and precipitated materials were collected after filtration and then dried and finally ashed. Based on the results from determined dry matter and ash in the sample and residue, respectively, EDOM was calculated.

***In vivo* determination of true metabolisable energy (TME_n)**
The method used is a procedure for determining digestibility of nutrients (McNab and Fisher, 1982, 1984; Fisher and McNab, 1987). In this bioassay technique, 50 g of the test feed is introduced into the crop of an adult rooster by means of a stainless steel funnel and tube. Each test feed is replicated among six roosters. Excreta are collected during 48-h period. These are dried, weighed and analysed. Endogenous energy or amino acid losses are determined in roosters kept under the same conditions, but glucose is fed in place of the test ingredient. These endogenous losses are used to calculate the true digestibility of the test nutrient.

Statistical analysis
Using statistical package STATISTICA (Data Analysis Software System), v.8.0. (2008), obtained EDOM and proximate analysis values were regressed against the *in vivo* ME results, and 3D graphs and correlation plots were generated. Coefficient of determination (R^2) and root mean square (RMS) were used as parameters for assessment of model adequacy.

RESULTS AND DISCUSSION

Correlation between TME_n and EDOM is presented in Figure 1. For the prediction of TME_n simple linear model with one parameter was proposed. The single coefficient with value of 0.191 was significant ($p < 0.001$). Coefficient of determination (R^2) of proposed model had fair value of 0.704, while RSD value was 0.127. The proposed model could be used for fast prediction of TME_n value, as no additional chemical analysis is required.

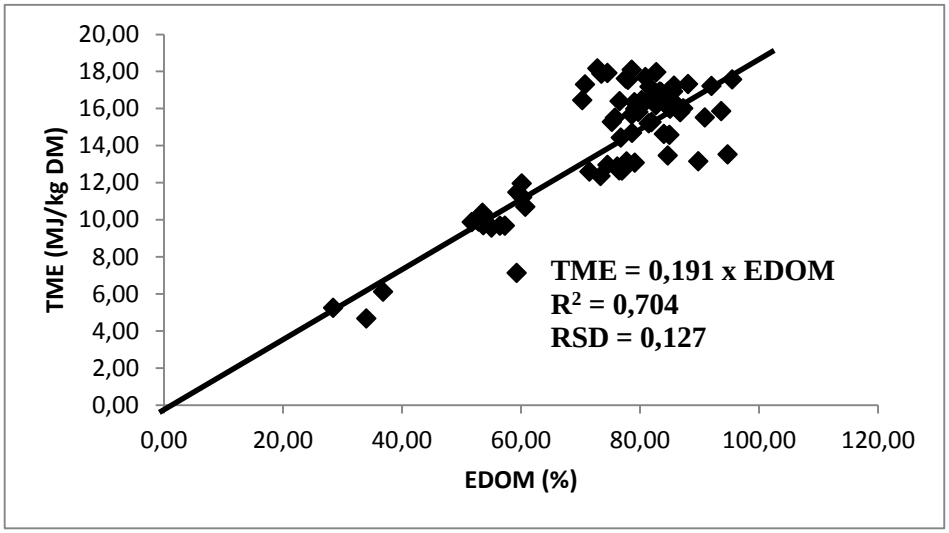

Figure 1. Correlation between TME_n and EDOM

In 3D graph (Figure 2) the correlation between TME_n and EDOM and fat is presented, i.e. linear model with two parameters was used for prediction of TME_n values. Both coefficients in model were significant ($p < 0.001$). Value of coefficient of determination (R^2) was 0.766, while value of RSD was 0.113.

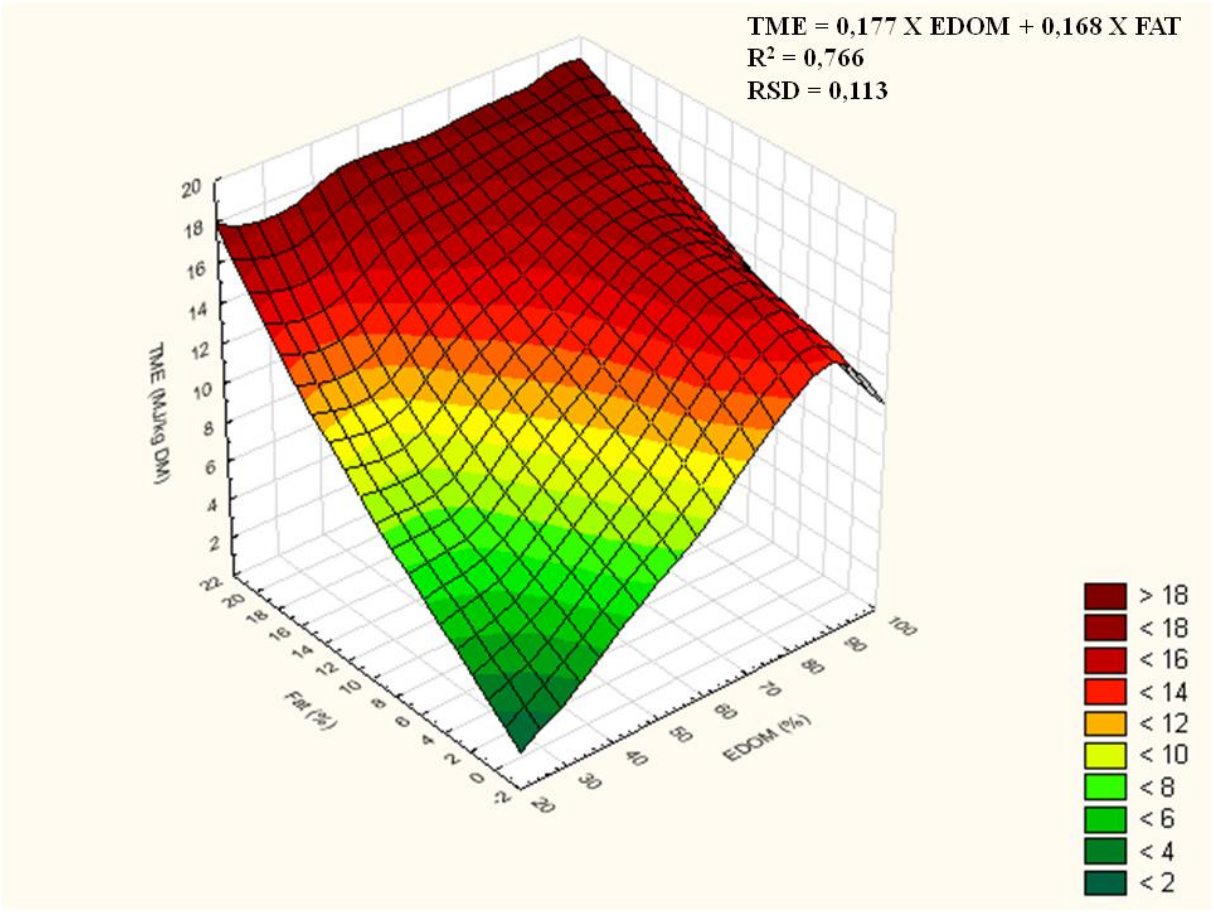

Figure 2. Correlation between TME_n and EDOM and TME_n and fat (3D graph)

Figure 3 presents correlation between TME and EDOM, fat and protein. Matrix graph was used for depicting validity of the proposed model. This graph is comprised of n x (n - 1) 2D graphs, where n is number of variables. Experimental values were presented with dots, while linear model was presented with linear trendline. All three coefficients in the model were significant ($p < 0.001$). Coefficient of determination (R^2) was 0.830, which according to Mayers and Montgomery (1995), can be considered as well fitting of data. RSD value of proposed model was 0.098.

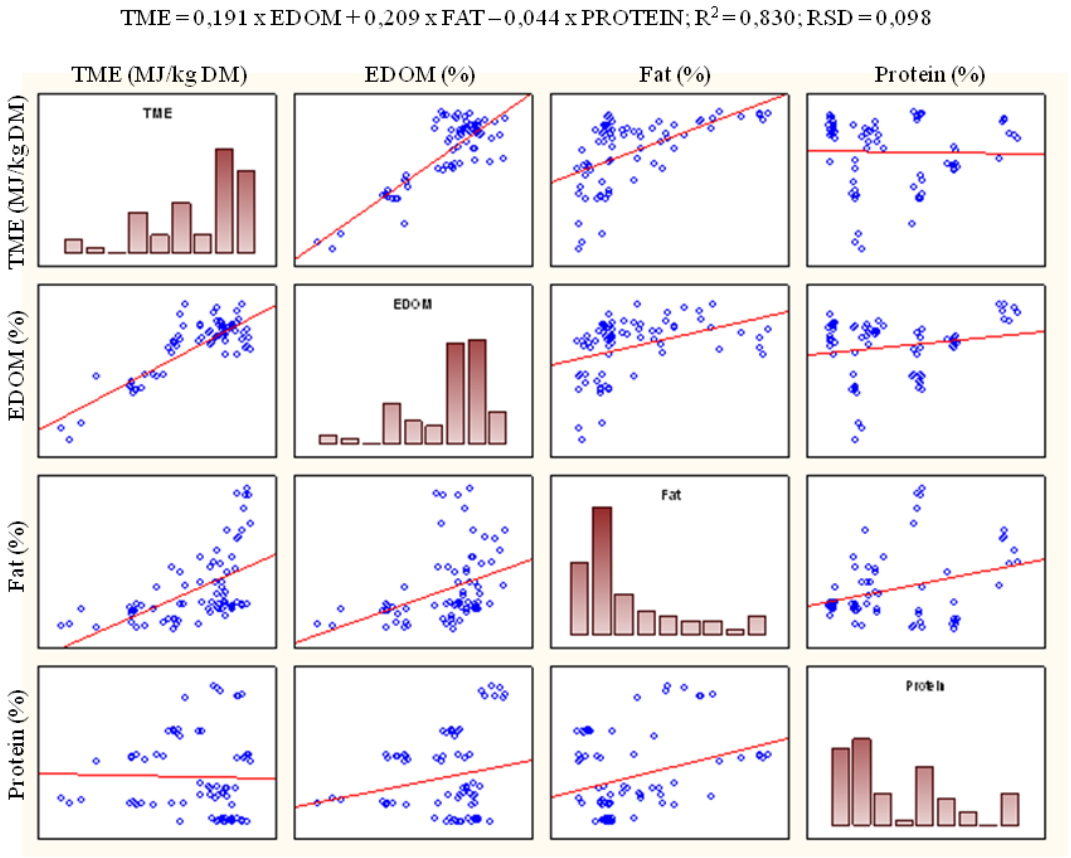

Figure 3. Correlation between TME_n and EDOM, fat and protein

Energy value of diets is of great importance for animal feed manufacturers and end users. The amount of available energy in feeds is described either by its metabolizable energy (ME) or by organic matter digestibility (OMD) (Pojić et al., 2008). Metabolisable energy is the most widely accepted value when expressing feed energy for poultry (Nwokolo, 1986; Farrell et al., 1991), however, its capability to estimate feed energy contents must be validated with *in vivo* determined values (Losanda et al., 2009, 2010).

In vitro methods used for predicting ME are rapid and not expensive (Farrel, 1999) as compared to *in vivo* determination of digestibility which is time-consuming and costly. Therefore, there has been a need for fast and reliable *in vitro* methods for determining nutrient digestibility in single feedstuffs for use in feed formulations and for control of complete diets (Boisen and Fernandez, 1997).

CONCLUSION

It can be concluded that the ME of feeds for poultry can be successfully predicted using the enzymatic procedure for determining the organic matter digestibility. Results of the statistical analysis showed that using only EDOM as a predictor is not as accurate as when the other variables from proximate analysis are included.

ACKNOWLEDGEMENT

The authors are grateful to the Ministry of Education and Science of Republic of Serbia for supporting this study through project III-46012, as well as to the Ministry of Science of Republic of South Africa for funding.

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THE IMPACT OF IRON AND MANGANESE CONCENTRATION IN WATER AND FEED ON THEIR PRESENCE IN TISSUES OF FATTENING BULLOCKS

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ABSTRACT: From January to June 2011 the content of iron and manganese in water, hay, complete feeds and muscular tissue, liver and kidneys of fattening bullocks after slaughtering was monitored with the aim of determining the correlation between the concentration of these elements in tissues and their quantity in water and complete feeds. The bullocks were bred on a mini farm in Kraljevo. Water and hay samples were collected on a fifteen-day basis. Ten samples of water, hay, complete feeds, muscular tissue, liver and kidneys were processed.
Apart from water, all the samples for the analysis were prepared by dry ashing and decomposition in a microwave oven (simultaneously), while Mn and Fe were determined by *atomic absorption spectroscopy* (AAS) (AOAC 1990) . The method of sample preparation did not affect the values of obtained results.
The average quantity of iron in water amounted 1.92 mg/L, 185.52 mg/kg in hay, 137.43 mg/kg in complete feeds, 38.87 mg/kg in muscular tissue, 67.60 mg/kg in liver and 78.83 mg/kg in kidneys. The average content of manganese was 1.57 x 10^{-2} mg/L in water, 76.01 mg/kg in hay, 12.64 mg/kg in complete feeds, 0.34 mg/kg in muscular tissue, 2.37 mg/kg in liver and 1.12 mg/kg in kidneys.
Our results showed that the intake of iron through water was significantly above MAC *(maximum allowable concentration)***,** whereas the quantity of manganese was substantially lower. The determined quantity of Fe in complete feeds was above minimal needs of bullocks as opposed to the quantity of Mn that did not comply with the proscribed limited quantity. The highest content of iron was determined in kidneys, and manganese had the highest value in bullocks liver.
Key words: *bullocks, feed, water, iron, manganese, tissues*

INTRODUCTION

When it comes to its content in animal organisms, iron is between micro and macro elements. A major part of iron can be found in blood hemoglobin and muscle myoglobin. The percentage of iron in hemoglobin is about 0.33%, while the remaining part of iron can be found in liver, spleen and kidneys. Iron in blood is an integral component of various important enzymes like hemoprotein enzymes (cytochromes) and flavonoid protein enzymes which have a very important role in oxidation processes of all cells. Iron is the key element in breathing process. As an integral part of hemoglobin, it enables transmission of oxygen and has the essential role in the reabsorption process.
Iron reabsorption takes place through intestine muscal which at the same time enables and controls its balance in organisms. Iron reabsorption in the digestion tract depends on the level of body reserves and the balance between the reabsorption process. The level of reserves is maintained. Some meal ingredients can affect the reabsorption process. It is believed that there is interdependence between the quantity of Ca, P and Fe and that reabsorption of one of them depends on its relative proportion in a meal. Fe reabsorption significantly decreases when the level of Ca is low and the quantity of phytic acid and P is high.
Fe excretion from organism is minimal and its quantity in serum is a typical indicator of the state of Fe enriched nutrition. The minimum Fe bullocks need from complete feeds is estimated to be about 20 mg/kg.
The first indicator of lack of Fe in food for animals is anemia. Anemia in bullocks occurs if they are solely fed with milk and no other food for a long time. As anemia problems in

humans are also related to the intake of Fe through beef, we found it important to determine the level of Fe in feed for bullocks, and in muscular tissue, liver and kidneys after slaughtering.
As opposed to other microelements, there is not much data available on the chemical composition of Mn or its combinations that can be found in animal organisms. Apart fromarginase in liver, which contains Mn as an essential component (Bach and Whitehouse, 1954) there is no metalloenzyme that contains a protein with a specifically determined quantity of Mn. However, Mn in vitro activates various enzyme systems, most commonly together with other divalent ions, magnesium in particular. The fact that Mn is concentrated in mitochondria implies that it is involved in partial regulation of oxidative phosphorylation. Manganese is not found in any specific organ or tissue, but its concentration in bones, liver, kidneys and pancreas is higher (1-3 mg/kg of fresh tissue) than in skeletal muscles (0.1-0.2 mg/kg).
Animal needs for Mn depend on animal species and the quantity of Ca and P in feeds. Estimation of Mn need is complicated because there is not much data on availability of Mn (in nutrition of poultry the availability is about 5 – 10 %), which means that fattening bullocks need at least 20 mg/kg of Mn. Copper has the key role in making use of iron, as Cu can be found in the enzyme ferroxidase which eases the process of iron release in mucous cells of intestines (Jovanovic et al., 2001). The quantity of Mn in hay is directly proportional to its content in soil, meaning that there are areas with deficit in Mn in hay because of its lack in soil. This is why Mn needs to be added to complete feeds for bullocks, taking care about its form. The most common additives are manganese oxide and manganese sulfate. If the relative biological utilization of manganese sulfate is 100%, then the utilization of manganese oxide is 60- 80%, manganese carbonate 25- 40% (Mc Dowell, 1992). Utilization of Mn from protein manganese or metionine Mn-chelate is, however, higher than that of manganese sulfate (Henry, 1995).

MATERIAL AND METHODS

The content of Fe and Mn in water, hay, complete feeds and muscular tissue, liver and kidneys after slaughtering of fattening bullocks was determined in this paper. The analysis included 10 samples of each of the examined parameters.
The samples of water, hay and complete feeds were taken from January to June 2011, on a fifteen-day basis, while the tissues were taken after slaughtering of cattle. The samples of water for the analysis were prepared by conservation (with nitrate acid) on the farm, and then they were concentrated in a laboratory. Hay samples were homogenized, chopped and burnt on a hot plate, and then calcinated in a calcinations oven at 550^{o}C. The residue after calcination was dissolved in HCl 1:1 and placed into 50ml vessels by redistilled water.
Complete feeds samples were homogenized in a homogenization mill “Cyclotec”, burnt on a hot plate and calcinated in a calcinations oven at 550^{o}C. The ash was dissolved in HCl 1:1, and determination of Fe and Mn in all the materials was performed by atomic absorption spectroscopy (AAS) method (Perkin Elmer 3300). These samples were destroyed in a microwave oven BEREGHOF - Speed (nitrate acid –hydrogen peroxide). Fe and Mn were determined in the same way with the aim to determine the impact of preparation method on the obtained result.

RESULTS AND DISCUSSION

The results of determination of Fe and Mn concentration in water, hay, complete feeds and tissues of fattening cattle are shown in tables 1 and table 2. The average quantity of Fe that cattle got through water amounted 1.92 mg/L with the interval of variation 0.47- 5.60 mg/l, while Mn amounted 1.57 x 10^{-2} mg/l. The current Rulebook on Hygienic Safety of Drinking Water does not limit the quantity of Fe, MAC for manganese is 0.050 mg/l. As the cattle farm is supplied with water from the Ibar alluvion, these results show that Mn quantity is almost

unchanged compared to determinations during 1987, 1988 and 1989. (Vukasinovic and Mihajlovic, 1990).
The results on Fe concentration in water for cattle were significantly higher than the results obtained by Vukasinovic and Mihajlovic R. (1990) who found that the concentration of Fe in the Ibar was 0.12 - 2.20 mg/l during 1987, 1988 and 1989. As the cattle farm and the town of Kraljevo are supplied from the Ibar alluvion, there is a significant increase of Fe level compared to the period ten years ago. Rajkovic et al. (2009) determined (indirectly, through aqueous lime scale) the presence of Fe and Mn in potable water in Belgrade. Their results show the presence of manganese in Vidikovac water supply network in the quantity of 0.040 mg/l, while the concentration of iron was 3.84 mg/l.

Table 1. Average concentration of Fe in water, hay and body tissues

Material	Measures of iron concentration variations (Fe)				
	n	x	Sd	Cv %	Iv (min-max)
Water (mg/l)	10	1.92	1.48	77.31	0.47-5.60
Hay (mg/kg)	10	185.52	83.25	44.87	66.44-302.41
Complete feeds (mg/kg)	10	137.43	18.073	13.15	103.32-158.87
Muscular tissue (mg/kg)	10	38.87	16.42	42.23	22.67-78.96
Liver (mg/kg)	10	67.60	13.10	19.38	45.27-88.37
Kidneys (mg/kg)	10	78.83	20.63	26.17	57.43-121.14

Average quantity of Fe in meadow hay from the Kraljevo area amounted 185.52 mg/kg with the variation interval of 66.44- 302.41 mg/kg of air dried hay. Sevkovic et al. examined the concentration of Fe (1989) in meadow hay from the Kraljevo area and they determined that it amounts 203.00 - 269.66 mg/kg of Fe, while the quantity of Fe in the hay from Pozega is insignificantly lower and it amounted 188.82- 247.28 mg/kg (Sevkovic et al.,1989). The average quantity of Mn in hay from Kraljevo amounts 76.01 mg/kg of air dried hay, with the variation interval of 32.08-100.16 mg/kg. The results we obtained are slightly higher that the results achieved by Sevkovic et al. (1989) who examined the concentration of Cu, Zn, Mn and Fe in meadow hay from Kraljevo and Pozega. They found that the concentration of Mn in meadow hay from Kraljevo amounted from 44.54 to 87.22 mg/kg, and that from Pozega was 68.42-143.95 mg/kg.
The average concentration of Fe in complete feeds for cattle amounted 137.43 mg/kg, with the variation interval of 103.32-158.87 mg/kg. The quantity of Fe was significantly above the quantity proscribed by the Rulebook on Animal feed Quality, which amounts 20.00 mg/kg. According to recommendations of NRC (2001), cattle need 2-3 mg of maganese per day and 10-20 mg of iron per day.

Table 2. Average concentration of Mn in water, feed and body tissues

Material	Measures of manganese concentration variations (Mn)				
	n	x	Sd	Cv %	Iv (min-max)
Water (mg/l)	10	1.57×10^{-2}	1.97×10^{-2}	125.15	0.002-0.070
Hay (mg/kg)	10	76.01	22.21	29.22	32.08-100.16
Complete feeds (mg/kg)	10	12.64	12.84	30.18	8.84-54.62
Muscular tissue (mg/kg)	10	0.34	9.70×10^{-2}	28.68	0.17-0.52
Liver (mg/kg)	10	2.37	0.32	13.69	1.81-2.86
Kidneys (mg/kg)	10	1.12	0.13	11.95	0.90-1.30

Average quantity of Mn in complete feeds amounted 12.64 mg/kg with variation interval of 8.84-54.62 mg/kg. The average concentration of Mn in complete feeds for the cattle was below the minimal needs stipulated by the Rulebook on Animal Feed Quality (“Official Gazette of the Republic of Serbia” number 4/10) in the amount of 20 mg/kg. As there is not much data on availability of manganese, it is difficult to determine the needs of cattle for manganese. There are few producers of complete feeds who use amino acid complexes as manganese source. Most of them use the salts of this element which are reabsorbed in small quantities (5-10%), while earlier research showed that organically chelated manganese is

reabsorbed better (80-90%) (McDowell, L.R.(1992). The reabsorption of manganese is increased by addition of EDTA to feed (Jovanovic et al., 2001).
Tables 1 and 2 show the results of examining the concentration of Fe and Mn in tissues of fattening cattle. The average concentration of Fe in muscular tissue amounted 38.87 mg/kg with variation interval of 22.67- 78.96 mg/kg, and Mn was 0.34 mg/kg with variation interval of 0.17-0.52 mg/kg. Livers of fattening cattle had the average concentration of 67.60 mg/kg of Fe with the variation interval of 45.27- 88.37, kidneys had 78.83 mg/kg with variation interval of 57.43- 121.14 mg/kg. The concentration of manganese in liver amounted 2.37 mg/kg with variation interval of 1.81-2.86 mg/kg, 1.12 mg/kg in kidney, with variation interval of 0.90-1.30 mg/kg. The determined quantity of Mn in liver was close to the quantity determined by Rogovski (1981), amounting 0.26 mg/100g.
Our results on the quantity of Fe in muscular tissue are higher than the results obtained by Rogovski (1981), finding the presence of Fe in the quantity of 3.00 mg/100g, while the concentration of Fe in liver determined by Rogovski was higher than our results (12.00 mg/100g). The concentration of Fe in kidneys was in accordance with the results obtained by Rogovski. The quantities of Fe determined by Djujic et al. (1981) in muscular tissue of cattle loin amounted 1.40 - 3.04 mg/kg, 1.22 - 2.56 mg/kg from flank and 1.42 - 3.57 in the meat from the head were significantly lower than the results we obtained.
According to the data on Fe and Mn concentrations in water, feed and tissues, we can conclude that the increase in concentration of examined minerals did not cause pathological changes that could result in impairment of general health of the examined cattle.

CONLUSIONS

Our examination provided the following results:
The samples of biological material for determination of Mn and Zn can be prepared from ash and by destruction in a microwave oven. The method of preparation does not affect the obtained results. The determined quantity of Fe in water for fattening cattle is significantly above the level determined by previous examinations, while the quantity of Mn did not exceed the MAC. The intake of Fe through complete feeds was significantly higher than the quantities proscribed by the current Rulebook, while the average quantity of Mn was below the minimum needs. The highest average quantity of Fe was determined in kidneys amounting 78.83 mg/kg, while the concentration of Mn in liver was 2.37 mg/kg.

ACKNOWLEDGEMENT

The work was realized within the project III 46012 co-financed by the Ministry of Education and Science, Gaverment of the Republic of Serbia.

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AMINO ACID COMPOSITION OF GILT'S DIETS ON FARMS IN VOJVODINA

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ABSTRACT: This paper presents the analysis of amino acid composition of the diet for gilts from 9 farms in Vojvodina, with a goal to determine protein and amino acid content in mixtures for gilts. The aim of this study was presentation of the situation on the ground in terms of quality amino acid composition of gilt's diets. The work aroused unreasonably high protein content, as well as the essential and non-essential amino acids content in diets, which are economically unjustifiable and high above the recommendations for gilts. Protein content was ranged from 21.36% to 10.61%. Essential and nonessential amino acids were highly correlated with the level of protein, as expected. The highest correlation with the protein level was observed for arginine r = 0.92, and lowest in case of alanine r = 0.68, while for lysine was r = 0.75.
Key words: *amino acids, gilts, diet, liquid chromatography*

INTRODUCTION

It has been known for many years that when swine are fed by diets that are deficient in protein they do not grow or reproduce normally (Pond, 1973; Baker and Speer, 1983). Therefore, proteins were considered to be essential dietary constituents. It is now clearly recognized, however, that it is not proteins per se but their components, amino acids, that are the essential ingredients. Young pigs gain weight when fed by diets containing no protein, but an appropriate mixture of amino acids (Shelton et al., 1950; Beeson et al., 1951; Mertz et al., 1952; Eggert et al., 1955; Chung and Baker, 1991). Furthermore, sows are able to maintain a normal pregnancy during the last 84 days of gestation when fed by a diet that contains crystalline amino acids as the sole source of nitrogen (Easter and Baker, 1976).
Proteins differ considerably in their nutritional value. Some, such as milk proteins, are high in nutritional value; others, such as sesame meal, are low. It is well established that the nutritional value (quality) of a protein is primarily dependent on its amino acid composition, especially the content of essential amino acid, and on the availability of the amino acids. A protein that contains a perfect balance of amino acids, both among the essential amino acid and between essential and non-essential amino acid, has been described as an *ideal* protein. This concept has been discussed by numerous authors (Fuller and Wang, 1990; Cole and Van Lunen, 1994; Lewis, 1995; Baker, 1997).
Estimation of the proportions of amino acids in ideal protein for growing swine have been derived from an examination of various types of data, including the composition of pig tissue, the composition of sows' milk, and combinations of individual estimation of amino acid requirements. The validity of assuming that there is one set of ideal proportions among amino acids for all stages of growth has been questioned (Lewis et al., 1977) because it is clear that the ideal pattern for maintenance differs from the ideal pattern for synthesis of new tissue. Therefore, the overall ideal pattern will change as the proportions of maintenance and new tissue synthesis change. In addition, there are changes in the amino acid content of pig tissue as the pig grows from birth to market weight (Kyriazakis et al., 1993; Susenbeth, 1995; Mahan and Shields, 1998).
In theory, any deviation of the pattern of amino acids in ideal protein will lead to a reduction in animal performance, at least in terms of the efficiency of dietary protein utilization. In practice, however, swine seem to be relatively tolerant to quite wide variations in the pattern

of amino acids, as long as all amino acid requirements are met. Nevertheless, if the dietary amino acid pattern deviates too far from the ideal, swine performance will be reduced. The negative effects caused by the ingestion of disproportionate amounts of amino acids have been classified into three main types: *toxicity*, *imbalance*, and *antagonism* (Harper et al., 1970).

Toxicities, characterized by the consumption of a large excess of an individual amino acid, are rare in practical swine nutrition. It could be caused only by misinformation or errors in mixing of a diet that included crystalline amino acids. Unfortunately, out of four amino acids that are currently available in a feed-grade form for growing-finishing pigs (lysine, methionine, tryptophan, and threonine), two (methionine and tryptophan) have the highest relative toxicity of the essential amino acids. In rats, the addition of 3% methionine to the diet is severely toxic (Benevenga and Harper, 1967). The rat's requirement in sulfur amino acids is 0.60%; thus the margin of safety is rather small. Swine seem to be particularly sensitive to excess of methionine (Baker, 1977; Edmonds and Baker, 1987a; Edmonds et al., 1987). In contrast, excesses of lysine (Lewis et al., 1986; Edmonds and Baker, 1987b; Edmonds et al., 1987) and especially threonine (Edmonds and Baker, 1987a; Edmonds et al., 1987) are well tolerated by young pigs.

Imbalances are also caused by excessive intake(s) of (an) amino acid(s), but usually the extent of the disproportion is less and there are no clear toxic features that are specific for the amino acid(s) involved. Imbalances are caused by the exacerbation of the deficiency of the most limiting amino acid, and they can be corrected by the appropriate addition of that amino acid. In an experiment by Wahlstrom and Libal (1974), as little as 0.2% added methionine reduced the performance of growing-finishing pigs, but this effect was alleviated by the addition of 0.2% lysine (the First limiting amino acid). In general, amino acid imbalances reduce feed intake with little or no effect on the efficiency of utilization of the First limiting amino acid (D'Mello, 1993).

In most practical swine diets, the amino acid "disproportion" of greatest concern is simply a deficiency of one or more amino acids. Feedstuffs with high protein content are usually relatively expensive and thus there is a tendency to limit their inclusion in diets. When the dietary protein content is inadequate to meet the requirements for all essential amino acids, swine performance will be restricted. The amino acid that is present in the least amount relative to its requirement is said to be the *First-limiting amino acid*, and the extent to which it is adequate will determine animal performance. If the deficiency of this amino acid is corrected, then the amino acid next lowest in relation to its requirement (second-limiting) will dictate animal performance. Information about which amino acids are most limiting in natural feedstuffs is important in formulating swine diets (Austin J. et al., 2000).

MATERIAL AND METHODS

Material

Acetonitrile (LC grade) and methanol (LC grade were purchased from Sigma-Aldrich (St. Louis, MO). Borate buffer, OPA and FMOC reagents and amino acid mixture standard solutions (10, 25, 100, 250 and 1000 pmol μl^{-1}) were obtained from Agilent Technologies (Waldbronn, Germany). Hydrochloric acid, used for preparation of 6 M and 0.1 M HCl, was obtained by Lach-Ner (Neratovice, Czech Republic). Sodium phosphate monobasic was purchased from Acros Organics (New Jersey, USA). LC grade water was produced by Heming ID-3 system (Belgrade, Serbia), while cellulose membrane filters (pore size 0.22 µm) were purchased from Agilent Technologies (Waldbronn, Germany).

Equipment

Vacuum tubes for hydrolysis (19 x 100 mm) were purchased from Pierce (Rockfors, IL). Hydrolysis and evaporation of the sample was derived using the React-Therm™ (Thermo Scientific, USA).

The analysis was performed using the Agilent 1260 Infinity Liquid Chromatography System, equipped with µ-Degasser (G1379B), 1260 binary pump (G1312B), 1260 Standard

Autosampler (G1329B), 1260 unit thermostat (G1316), ZORBAX Eclipse-AAA column and 1260 DAD detector (G1315C).

Samples

Samples were collected from nine farms in Vojvodina, with a goal to determine protein and amino acid content in mixtures for gilts. Samples of mixtures for feeding gilts were milled in a laboratory mill with a sieve size of 0.5 mm. 0.1 to 1.0 g of sample was measured (equivalent to the nitrogen content of 10 mg) in a vacuum hydrolysis tube, and then hydrolyzed with 7 ml of 6M HCl. Hydrolysis was carried out with React-Therm^{TM} apparatus with constant stirring for 6 hours at 150 °C. After hydrolysis, samples were allowed to reach the room temperature and then evaporated to dryness using the React-Therm^{TM} at 70 °C in a stream of nitrogen. The residue was quantitatively transferred into a 50 ml volumetric flask with 0.1 M HCl. The solution was filtered through quantitative filter paper, and then additionally filtered using a cellulose membrane filter (pore size 0.22 µm).

Table 1. Injector programs

Injector program
Draw 2.5 µL from vial 1 (borate buffer)
Draw 0.5 µL from sample (position X)
Mix 3 µL .in air., max speed, 2x
Wait 0.5 min
Draw 0 µL from vial 2 (water, uncapped vial)
Draw 0.5 µL from vial 3 (OPA)
Mix 3.5 µL .in air., max speed, 6x
Draw 0 µL from vial 2 (water, uncapped vial)
Draw 0.5 µL from vial 4 (FMOC)
Mix 4 µL .in air., max speed, 6x
Draw 32 µL from vial 5 (water)
Mix 18 µL .in air., max speed, 2x
Inject

HPLC determination

Chromatography conditions were in accordance with the Agilent method (Henderson et al., 2000) with the exception of mobile phase A preparation where 5.678 g Na_2HPO_4 was dissolved in 1 L water and then adjusted with 6M HCl to pH 7.8. Hydrolyzed samples and standard mixtures of amino acids were automatically derivatized with OPA and FMOC reagents using the appropriate injector program (Table 1). After derivatization, an amount equivalent to 0.5 µl of sample or standard mixture was injected on ZORBAX Eclipse-AAA column (5 µm, 150 × 4.6 mm) at 40 °C, with detection at λ1 = 338 nm and λ2 = 262 nm. Mobile phase A was 40 mM Na2HPO4, adjusted with NaOH to pH 7.8, and mobile phase B acetonitrile / methanol / water (45:45:10 v:v:v). Separation was performed at a flow rate of 2 ml min^{-1} with a gradient program (Table 2) during the 26 min period of analysis.

Table 2. Gradient mobile phase

Time (min)	% B
0	0
1,9	0
18,1	57
18,6	100
22,3	100
23,2	0
26	0

B - Percent mobile phase

All data were analyzed to test the correlation and for analysis Statistica 10 (Statasoft 2010) was used.

RESULTS AND DISCUSSION

The results of analysis of amino acids and protein in the diets for gilts from nine farms in Vojvodina are shown in Table 3. On the basis of shown values, it can be seen that only a mixture from farm no. 7 does not meet the criteria established by the "Regulations on the quality of feed". According to the "Regulations on the quality of feed" the minimum level of protein in the mixture should be 13%, and minimum level of lysine should be 0.55% (Pravilnik o kvalitetu hrane za životinje, 2010).

Table 3. Essential amino acids and protein content in diets for gilts from nine farms in Vojvodina

%	1	2	3	4	5	6	7	8	9
Protein	20.37	18.44	18.83	21.36	19.96	18.50	10.61	17.69	14.76
Lysine	1.173	1.130	1.131	1.575	2.262	1.212	0.503	1.020	0.769
Methionine	0.236	0.263	0.256	0.319	0.309	0.269	0.187	0.213	0.244
Threonine	0.675	0.589	0.567	0.729	0.916	0.614	0.341	0.531	0.434
Arginine	1.240	1.169	1.239	1.342	1.613	1.231	0.560	1.140	0.786
Histidine	0.610	0.592	0.645	0.600	0.952	0.538	0.205	0.577	0.391
Isoleucine	0.815	0.886	0.848	0.834	1.347	0.895	0.413	0.814	0.594
Leucine	1.649	1.655	1.639	1.610	2.446	1.696	0.179	1.555	1.290
Phenylalanine	0.973	0.983	0.932	0.980	1.417	0.948	0.467	0.876	0.663
Valine	0.842	1.048	1.038	0.860	1.521	0.938	0.458	0.846	0.618

Results are given as mean, n = 3 samples; Farms were signed by numbers because of discretion policy not to publish the identity of the farm

In diet from farm no. 7, where was established the lowest level of protein, lysine content was 0.50 which is slightly lower than in the "Regulations on the quality of feed". Consequently, approximately 60 g of ileac digestible lysine/MJ of DE was required to optimize performance in gilts with high genetic potential for lean gain, irrespective of the feed intake level. In addition, a factorial model to estimate the lysine/energy requirements is proposed (Bikker et al., 1994).

Table 4. Non-essential amino acids content in diets for gilts from nine farms in Vojvodina

	1	2	3	4	5	6	7	8	9
Aspartic acid	1.797	1.720	1.632	1.877	2.611	1.811	0.806	1.581	1.183
Glutamic acid.	4.000	3.635	3.824	3.848	5.294	4.023	2.101	3.913	2.688
Serine	0.868	0.581	0.545	0.865	0.878	0.606	0.300	0.507	0.504
Glycine	0.811	0.846	0.911	0.913	1.243	0.874	0.433	0.830	0.553
Alanine	0.970	1.038	1.016	1.027	1.462	1.032	0.558	0.919	0.772
Tyrosine	0.493	0.373	0.388	0.476	0.595	0.495	0.224	0.399	0.338
Cysteine	0.114	0.056	0.057	0.128	0.076	0.072	0.038	0.067	0.058

Results are given as mean, n = 3 samples; Farms were signed by numbers because of discretion policy not to publish the identity of the farm

Results based on the feed intake observed in study, where the high-lean-growth gilt requires at least 22 g/d total lysine intake from 34 to 72.5 kg to maximize CP accretion (Hansen and Lewis, 1993).

The results of analysis of non-essential amino acids in diets for gilts, also with the nine most important farms in Vojvodina, are shown in Table 4. It is evident that the diet from farm no. 7 shows lower values than other farms, which was the case with the previous table in terms of protein levels and essential amino acids.

Correlation analysis (Table 5) between, protein and amino acid levels for a given farm indicates a significant difference ($p < 0.05$).

Table 5. Correlation between protein and essential amino acids

	Lys	Met	Thr	Arg	His	Ile	Leu	Phe	Val
Protein	0.75[a]	0.76 [a]	0.84 [a]	0.92 [a]	0.81 [a]	0.74 [a]	0.85 [a]	0.82 [a]	0.68 [a]

[a]*significant difference (p<0,05)*

Table 5, shows high correlation of protein levels and arginine r = 0.92, when it comes to the essential amino acids, and also a high degree of correlation was noted with almost all the essential acids (Table 5).

Table 6. Correlation between protein and non essential amino acids

	Asp	Glu	Ser	Gly	Ala	Tyr	Cys
Protein	0.83	0.83	0.87	0.82	0.78	0.85	0.75

[a]*significant difference (p<0,05)*

Also in Table 3 are evident unreasonably high values of protein content that reach up to 21.36%, as opposed to farm no. 4. If we notice the values for lysine, also shown in the same table, it can be seen that all values are unreasonably high, and some even more than double and reach up to 2.262% (sample of diet from farm 5), which is more than three times higher compared to the Regulations.
The values in Table 6 regarding the non-essential amino acids (p<0.05) were also highly correlated with the level of protein in the mixture, with many small mutual deviations.
The correlation between lysine and protein content (r = 0.75) is shown in Figure 1 and it indicates a significant but not very high correlation as it is the case with some other amino acids. Reasons for this may be related to the fact that the high level of synthetic lysine was added and that a good portion comes from soybeans.

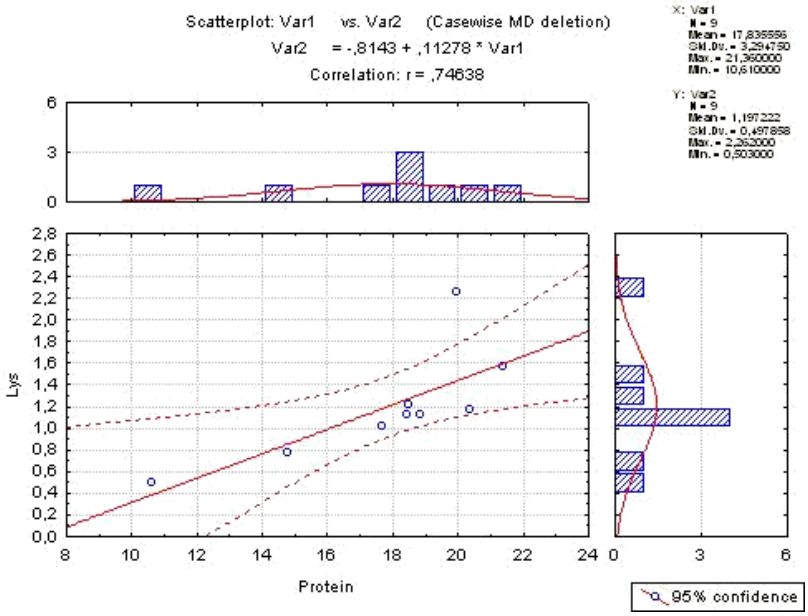

Figure 1. Correlation between lysine and protein content

CONCLUSIONS

Results of amino acid composition analysis of swine feed show less variation in terms of amino acid content which is primarily a consequence of the structure of the mixture. Since the First limiting amino acid in the diet of pigs is lysine, it is particularly necessary, in addition to other amino acids, to pay attention to its level in the diet.
Levels of protein in samples of analyzed diets for gilts on most farms are unjustifiable high. That level is higher than the level of requirements provided by the "Regulations on the quality of feed", compared to the level of protein and essential amino acids. From the point of meeting the requirements of the ideal protein diet, it is much more efficient to achieve with the levels of proteins that are closer to the needs of the given categories with the addition of synthetic amino acids.

One of the recommendations is that in the preparation of meals as a laboratory controlled mixtures, needs to be devoted more attention to amino acid composition and essential amino acids ratio than the protein content.

ACKNOWLEDGEMENTS

The work was financially supported by the Ministry of Science and Technological Development, Republic of Serbia (Project No 31081).

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SOURCES AND EFFECTS OF DIFFERENT LEVELS OF OIL ON PRODUCTION PERFORMANCE AND BROILERS CARCASS QUALITY

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ABSTRACT: This paper investigates the effect of soybean oil, flax and rapeseed on production parameters and broilers carcass quality. At the beginning of fattening six groups of 40 one-day-old chick's hybrid Ross 308 were formed, with five replications. The control group was based on two levels of soybean oil, 4% and 8% and in the experimental groups were included 4% and 8% flax, or 4% and 8% rapeseed oil. Experiment lasted for 42 days. At the end of fattening period, 10 chickens from each group were sacrificed for the purpose of testing carcass quality. Including various types of oils in the diet did not result in statistically significant differences in body weight of chickens. At the end of the experiment, the control has achieved a body mass of 2704.0 g and 2695.0 g, experimental group with flax oil 2734.0 g and 2671.0 g and with rapeseed 2735.0 g and 2670.0 g. Feed conversion was the lowest in groups with rapeseed oil and the highest in groups with flaxseed oil. The difference in the carcass quality of control and experimental groups were not significant. The highest amount of abdominal fat (31,9g) was in the group with addition of 8% flax oil, and the lowest in the group with soy bean oil in same amount (22g) Treatments with 4% of oil, have almost the same amount of abdominal fat.
Key words: *nutrition, flax oil, rapeseed oil, soy oil, chicken, carcass quality*

INTRODUCTION

At the end of the last century the market was buying chickens with highest weight and highest efficiency for major body parts. Nowdays, there is a need for chicken meat with the altered structure of fat tissue rich in poliunsaturated omega-3 fatty acids, in order to reduce the number of people with cardiovascular illnesses. For that reason, the researchers are questioning the influence of the type and amount of oil on the growth intensity, feed utilization efficiency, carcass quality, and the chicken meat quality. Lopez-Ferrer et al., (2001) established that 2 and 4% flax oil in broilers mixtures, with addition of of 8% affects the fatty acid content of the tissue, while the production paremeters showed smoler diferences between treatments. Differences in yield of carcass mass between groups, were not significant ($P>0.05$). However, the results given by Bartos et al., (2004) show negative influence of the addition of 6% of flax oil in the mixture for broilers on the carcass quality. When the flaxseed meal was added in the amount of 5, 10 and 15% in the broilers chicken nutrition, during the first two weeks, growth depresion was not record. After that period differences in body mass are becoming more and more prominent so that at the end of the 6 week chickens were on the treatment of 15% of falxseed meal with recorded lower body mass for 8%, statistically significant ($P<0.05$) in accordance with the control group. Feed consumption was decreased, and conversion increased. The body mass was also decreased with the increase of participation of flaxseed meal, while the content of linoleic acid in red and white meat increased without the influence on sensor quality of meat (Mridula et al., 2011). The usage of the expeller of rapeseed meal in amount of 0 to 40% in the mixtures based on corn and soybean meal in broiler nutrition, did not significantlly ($P>0.05$) influence on the heart, kidney and thiroide gland mass, nor on the concentration of trijodthyronin and tetrajodthyronin, but it resulted in decreased growth and altered liver function (Woyengo et al., 2011). Using the oils with poliunsaturated fatty acids in broiler nutrition, to similar results

came Božić (1997), Antogovani et al., (2006), Azain (2004), Alparslan and Ozdogan (2006), Azman et al., (2005), Balevi and Coskun (2000), Bartos et al., (2004), Lopez-Ferrer et al., (1999), Stanaćev (2009), Talebali and Farzinpour (2005), Krasicka et al., (2000), Fouladi et al., (2008), Nelson (2005), Stanaćev et al., (2011). Having in mind the aforementioned, the aim of the research was to examine the productive parameteres and carcass quality of broilers chicken fed with different amounts of soy, flax and rapeseed oil.

MATERIAL AND METHODS

The researches conducted in productive conditions on the experimental farm »Pustara« in Temerin on the floor system of breeding. At the begining of fattenig period six groups with 40 one-day-old chicken of the hybride Cobb 500 were formed. The experiment is conducted in five repetitions, on the 200 chickens per treatment. Three mixttures with 21, 20 and 18% of protein where used for nutrition. First 14 days there was a preparation period for chickens, in which all groups were fed by starter mixture of standard content and quality. Then, for 21 days, grower mixtures with different source and oil amount were used. For the last 7 days, the chicken were fed by finisher mixtures with the same oil addition. The control group was fed with mixture based on 4 and 8% of soy oil, and in experimental groups there was 4 and 8% of flax and rapeseed oil (Table 1).Mixtures were izoprotein and izoenergetic. During the experimental period, that lasted for 42 days, the chickens were fed and watered *ad libitum*, and microclimatic conditions were regularly controlled. The control of body mass and the feed consumption was made every 7 days. At the end of the experiment, after 12 hours of starving, 10 chickens were taken from every group (5 females and 5 males) of avrerage body mass, marked with stamps and sacrificed for the needs of carcass quality research. After that there was debleeding, boiling, defeathering, taking out internal organs, and cooling. After that, clasically processed carcass was measured and cut on basic anatomic parts and measured (Regulations: Sl. List SFRJ, No.1/81 and 51/88). Marking was made based on yield and mass of certain parts of the carcass. For correct interpretation, given results were processed by the appropriate statistic methodes ANOVA (STATISTICA 9.1, StatSoft, Inc. 2010).

Table 1. Experiment with chicks as planed

Group	**I**	**II**	**III**	**IV**	**V**	**VI**
	Control, Soy oil	Control, Soy oil	Flax oil	Flax oil	Rapeseed oil	Rapeseed oil
In grower	4%	8%	4%	8%	4%	8%
In finisher	4%	8%	4%	8%	4%	8%

RESULTS AND DISCUSSION

Based on the results given, it can be stated that introduction of different kinds and amounts of vegetable oil in nutrition of broilers did not affect the intensity of growth (Table 2). During the preparation period, the chickens had almost equal body mass per group. In the fifth week of the first experimental period it can be noticed that there is a very small depression in V group, on the treatment with 4% of rapeseed oil compered to control group. At the end of the experiment, the depression of growth is insignificantlly ($P>0.05$) marked in IV and VI group in the amount of 1.04 and 0.89%, while the III and V group more superior for 1.22 and 1.15% compared to the control group. With the analysis of variance and Duncan test, it is determinated that there are no statistically significant ($P>0.05$) differences among the groups.

Table 2. Chicken body weight, g

Week – experiment phase	Groups					
	I	**II**	**III**	**IV**	**V**	**VI**
	4% soy oil	**8% soy oil**	**4% flax oil**	**8% flax oil**	**4% rapeseed oil**	**8% rapeseed oil**
Initial weight	42	42	42	42	42	42
1	185	185	183	190	187	190
2	438	448	451	446	455	450
Index, %	**100.00**	**100.00**	**102.97**	**99.55**	**103.88**	**100.45**
3	986	967	992	999	994	976
4	1456	1407	1524	1518	1515	1566
5	2131	2055	2148	2093	2121	2085
Index, %	**100.00**	**100.00**	**100.80**	**101.85**	**99.53**	**101.46**
6	2703	2697	2736^{ns}	2669^{ns}	2734^{ns}	2673^{ns}
Index, %	**100.00**	**100.00**	**101.22**	**98.96**	**101.15**	**99.11**

ns-not significant (P>0.05)

When the feed conversion is in question, there is a noticable increase of 5.03% in III and 2.07% in IV group on the treatment of flax oil, while the V and VI group on the treatment of rapeseed oil, had more efficient utilization of feed for 0.52% and 2.69% (Table 3).

Table 3. Feed converzion, kg

Week – experiment phase	Groups					
	I	**II**	**III**	**IV**	**V**	**VI**
	4% soy oil	**8% soy oil**	**4% flax oil**	**8% flax oil**	**4% rapeseed oil**	**8% rapeseed oil**
1	1.129	1.145	1.134	1.116	1.127	1.075
2	1.34	1.327	1.291	1.346	1.354	1.331
Index,%	**100.00**	**100.00**	**96.34**	**101.43**	**101.04**	**100.30**
3	1.365	1.353	1.385	1.422	1.408	1.406
4	1.477	1.468	1.41	1.494	1.453	1.486
5	1.543	1.519	1.589	1.666	1.516	1.543
Index,%	**100.00**	**100.00**	**102.98**	**109.68**	**98.25**	**101.58**
6	1.731	1.784	1.818	1.821	1.722	1.736
Index,%	**100.00**	**100.00**	**105.03**	**102.07**	**99.48**	**97.31**

Average values of carcass mass, yield and mass of certain carcass parts, as well as their relative part in the mass of the bare carcass showen in the table 4, direct to the point that there are very small difernces in all examined parameters and that the influence of feed treatment on yield of carcass is not statistically significant (P>0.05).
In the mass of bare carsass, white meat presents the biggest part with 31.90%-34.04%, then back with 18.25-18.93%, drumstick with 13.42-13.88% and thight with 9.56-11.02%, while the wings are the smallest part with 8.15-9.12%.

Table 4. Chicken troops quality at 42 day of age

Group	I	II	III	IV	V	VI
	4% soy oil	**8% soy oil**	**4% flax oil**	**8% flax oil**	**4% rapeseed oil**	**8% rapeseed oil**
Chicken body weight, g						
Before slaughter, g	2726	2630	2724	2709	2724	2712
Troup weight, g	2270.56	2198.16	2315.32	2313.85	2286.11	2277.63
Yield, %	83.29	83.58	85.00	85.41	83.92	83.98
Important parts weight, g						
Wings	201.9	2004	202.9	188.6	203.2	204.1
Tight	236.2	239.0	221.4	251.5	242.8	251.0
Drumstick	304.6	299.5	317.6	316.5	317.4	311.0
Brest	766.5	711.1	788.1	747.4	736.5	726.6
Back	414.3	403.1	426.1	438.0	419.4	426.0
Total	1923.5	1853.1	1956.1	1942.0	1919.3	1918.7
Index, %	**100.00**	**100.00**	**101.69**	**104.80**	**99.78**	**103.54**
Les important parts weight, g						
Abdominal fat	25.3	22.0	25.8	31.9	26.8	24.7
Liver	49.94	50.96	48.57	51.3	48.9	52.83
Heart	12.00	13.04	11.35	13.07	12.46	13.28
Craw	35.52	37.86	40.7	41.68	43.55	34.32
Head	56.2	53.6	57.6	58.8	59.1	56.8
Neck	86.2	79.6	90.2	87.2	88.8	85.9
Legs	81.9	88.0	85.0	87.9	87.2	91.1
Total	347.06	345.06	359.22	371.85	366.81	358.93
Index, %	**100.00**	**100.00**	**103.50**	**107.76**	**105.69**	**104.02**
Relative share of carcass parts, %						
Wings	8.89	9.12	8.76	8.15	8.89	8.96
Tight	10.40	10.87	9.56	10.87	10.62	11.02
Drumstick	13.42	13.63	13.72	13.68	13.88	13.65
Breast	33.76	32.35	34.04	32.30	32.22	31.90
Back	18.25	18.34	18.40	18.93	18.35	18.70

CONCLUSIONS

Based on the resultes given above, it can be concluded that utilization of 4 and 8% of flax and rapeseed oil did not show significant differences concerned with productive parameters and carcass quqlity compared to control group that had a mass of 2703g and 2697g, and experimental groups in order 2736, 2669, 2734 i 2673g. Feed conversion was increased for 5.03% in III and 2.07% in IV group on the treatment of flax oil, whike the V and VI group on the treatment of rapeseed oil had more efficient utilization of feed for 0.52% and 2.69%.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR-31033 “Sustainable conventional and revitalized traditional production of poultry meat and eggs with added value”, financed by the Ministry of Science and Technological Development, Republic of Serbia.

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FIBERS AND CELL WALL CONTENT AND IN VITRO DIGESTIBILITY OF DIFFERENT MAIZE HYBRIDS

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ABSTRACT: Analysis of the fiber or cell wall present in forages is of major concern in ruminant nutrition because diets often contain large amounts of forage, and the fiber fraction affects both feed intake and animal performance. Research has revealed that plant genetics can affect the quality and digestibility of whole plant maize silage. All carbohydrates in plant feeds are grouped into: 1. Structural carbohydrates (carbohydrates of cell walls) including NDF (neutral detergent fibers-hemicellulose+cellulose+lignin), ADF (acid detergent fibers-cellulose+lignin), ADL (lignin), and 2. Non-structural carbohydrates-NFC (carbohydrates located inside the plant cell) made of starch, sugars and pectin. The aim of this present study was to observe quality parameters of ZP hybrids biomass with different genetic background for silage and to determine the relationship of these parameters, as well as, their effects on the digestibility of maize biomass dry matter. The contents of lignocelluloses fraction were determined by the modified Van Soest detergent method while *in vitro* digestibility of the whole plant was done by the Aufréré method. Obtained results showed that the NDF, ADF and ADL contents in the whole maize plant of the observed different ZP hybrids varied from 42.6% to 50.9%, 19.3% to 25.7%, and 1.6% to 2.5%, respectively. The difference in the digestibility of the dry matter of the whole plant between hybrids amounted to 10.4%. The differences in the contents of lignocelluloses fraction affected the differences in digestibility of dry matter.
Key words: *NFC, NDF, ADF, ADL, digestibility, maize hybrids*

INTRODUCTION

Maize is one of the most important naturally renewable carbohydrate raw materials. As a high-yielding carbohydrate plant, maize is very competitive in relation to other cereals (Radosavljevic et al., 2010). All carbohydrates in plant nutrients are grouped into: I structural carbohydrates (carbohydrates of cell walls), which include NDF (neutral detergent fibers - hemicellulose+cellulose+lignin), ADF (acid detergent fibers - cellulose+lignin), ADL (lignin) and II nonstructural carbohydrates - NFC (carbohydrates present in the plant cell content) that are made of starch, sugars and pectin (Jovanović et al., 1993). However, according Polakova et al. (2010) the NFC of substances comprises a diverse group of substances in terms of their composition and nutrient contents, excluding carbohydrates inherent to NDF. The NFC group of substances includes organic acids, monosaccharides, oligosaccharides, fructans, starch, pectin substances and □-glucans. There are numerous confirmations that the feeding ration with a high NFC content ferment and produce a great quantity of propionic acid in the rumen, which results in a higher insulin concentration in blood, i.e. in animal weight gain. Feeding rations rich in digestible fibers (hemicellulose and cellulose) produce acetic acid in the rumen and increase the growth hormone level in blood, which results in the increased milk production (Jovanović et al., 1993). Analysis of the fiber or cell wall present in forages is of major concern in ruminant nutrition because diets often contain large amounts of forage, and the fiber fraction affects both feed intake and animal performance. Research has revealed that plant genetics can affect the quality and digestibility of whole plant maize silage (Jung, 1997). Considering all stated, as well as, the fact that the digestibility does not depend on the energy concentration, the objective of this present study was to observe quality parameters of the whole plant of ZP hybrids with different genetic background for silage and to determine the relationship of these parameters, as well as, their effects on the digestibility of maize biomass dry matter.

MATERIALS AND METHODS

The hybrids of the FAO maturity group 100-700 (ZP 161, ZP 388, ZP 434, ZP 555, ZP 677, ZP 704) were used in this study. The two-replicate trail was set up according to the randomized complete-block design in the experimental plot of the Maize Research Institute, Zemun Polje. The experimental plot size amounted to 21m^2, while sowing density was 60,000 plants per hectare. Plants of each replicate were harvested in the full waxy maturity stage from the area of 7m^2 (two inner rows), and yields of fresh biomass of the whole plants, plants without ears and ears were estimated. Five average plants per replicate were selected for further tests. Samples of the whole plants were cut and dried at 60^{o}C for 48h. In order to determine the content of dry matter the whole plant samples were ground in the 1-mm mesh mill. Then, the analysis of the absolute dry matter was done on the oven dry basis (105^{o}C for 12 h) in order to estimate the total dry matter. Moreover, the analysis of the content of forage fibers (NDF, ADF, ADL, cellulose, hemicellulose) was performed by the modified Van Soest detergent method (Van Soest*,* 1963)*.* The method was modified by Mertens (1992). *In vitro* digestibility of the whole maize plant was done by the Aufréré method *(*Aufréré, 2006). This method is based on the hydrolysis of proteins of the whole plant in the pepsin acid solution (Merck 2000 FIP u/g Art 7190) at 40^{o}C for 24 h, and then on the hydrolysis of carbohydrates in the cellulase solution (cellulose Onozuka R10) in duration of 24 h. Methods applied in order to determine basic chemical content of the maize plant samples (contents of ash, protein and fat) were described in previously published paper (Radosacljević et al., 2000). The NFC content in the whole plant samples is calculated according to the formula: (%) NFC = 100%DM – ((%) crude protein + (%) NDF + (%) fat + (%) ash) (Polakova et al., 2010).

Statistical analysis of data

Data reported for quality parameters of ZP hybrids biomass was assessed by analyses of variance (ANOVA) and LSD multiple test was used for any significant differences at the P<0.05 level between the means. All the analyses were conducted using statistical software package STATISTICA 8.1. (StatSoft Inc. USA).

RESULTS AND DISCUSSION

Table 1 presents the NFC content of the whole plant and evaluation of significance of difference between the observed ZP maize hybrids. The results showed that the NFC content in the whole maize plant of the observed different ZP hybrids varied from 33.20% to 41.22%. The difference in the NFC content of the tested ZP hybrids was 8.02%. Differences in the contents of NFC were statistically significant between hybrids ZP 388, ZP 555, ZP 704, and between these and the ZP 161, ZP 434 and ZP 677. Differences in the contents of NFC were not statistically significant between the ZP 161, ZP 434 and ZP 677.

Table 1. Content of NFC of the whole plant of the ZP maize hybrids

Hybrids	NFC (%)
ZP 161	39.93^d
ZP 388	41.22^a
ZP 434	33.20^d
ZP 555	39.63^b
ZP 677	34.33^d
ZP 704	38.14^c
$LSD_{0.05}$	1.29

Means in the same column with different superscripts differ (p<0.05)

Data on the content of NDF, ADF, ADL, hemicellulose and cellulose are presented in Table 2. The results showed that the NDF, ADF, ADL, hemicellulose and cellulose contents in the whole maize plant of the observed different ZP hybrids varied from 42.57% to 50.84%,

19.32% to 25.74%, 1.63% to 2.51%, 23.26 to 26.07% and 17.69 to 23.62%, respectively. The differences in the content of NDF, ADF, ADL, hemicellulose and cellulose among observed ZP hybrids were 8.27%, 6.42%, 0.88%, 2.82% and 5.83%, respectively. Statistically significant differences in the NDF content of tested ZP hybrids were found, except between the ZP 434 and ZP 677, ZP 555 and ZP 704. Differences in ADF content were statistically significant except between hybrids ZP 161 and ZP 704. Statistically significant differences in the ADL case were found only between hybrids ZP 161 and ZP 555. The difference in ADL content was statistically significant only between hybrids ZP 161 and ZP 388. In other ZP hybrids these differences were not significant. Differences in hemicellulose content were not statistically significant between the ZP 161 and ZP 434 and ZP 677, and between ZP 388, ZP 555 and ZP 704. Statistically significant differences in cellulose content in the examined maize hybrids were found. The differences in cellulose content were not statistically significant between the ZP 161 and ZP 704.

Table 2. Content of forage fibres of the whole plant of the ZP maize hybrids

Hybrids	NDF (%)	ADF (%)	ADL (%)	Hemicellulose (%)	Cellulose (%)
ZP 161	49.08^{b}	23.73^{c}	2.51^{a}	25.35^{ab}	21.22^{c}
ZP 388	42.57^{d}	19.32^{e}	1.63^{b}	23.25^{c}	17.69^{e}
ZP 434	50.84^{a}	24.77^{b}	2.19^{ab}	26.07^{a}	22.58^{b}
ZP 555	45.35^{c}	22.05^{d}	1.63^{b}	23.26^{c}	20.42^{d}
ZP 677	50.83^{a}	25.74^{a}	2.12^{ab}	25.09^{b}	23.62^{a}
ZP 704	46.52^{c}	22.95^{c}	1.93^{ab}	23.57^{c}	21.02^{c}
$LSD_{0.05}$	1.22	0.84	0.64	0.77	0.59

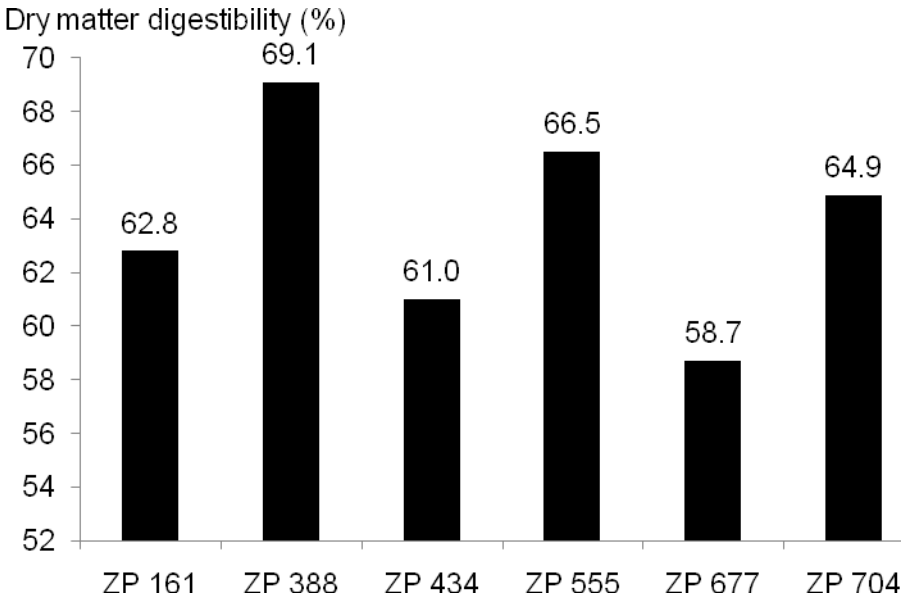
Means in the same column with different superscripts differ ($p<0.05$)

Considering the crucial effect of the NFC and forage fibers on the digestibility of the whole maize plant dry matter, the correlation dependence between the content of these components and the dry matter digestibility was observed (Table 3). A highly significant correlation between NFC content and dry matter digestibility of whole plant maize hybrids (r = 0.90), and a significant negative correlation between the content of NFC and the content of NDF, ADF, ADL, hemicellulose and cellulose were established (r = - 0.97, r = -0.90, r = -0.83, r = -0.96, r = -0.84). A very significant negative correlation was determined between the digestibility and NDF, ADF, hemicellulose and cellulose content (r=-0.96, r=-0.98, r=-0.79, r=-0.97), and significant negative correlation between the ADL content and the dry matter digestibility. Wermke (1986) ascertained a significant negative correlation (r=-0.67) between the digestibility and the ADF content, as well as, between the digestibility and lignin (r=-0.95). Burritt et al. (1985) studied three grass species and found high correlation dependence between the dry matter digestibility and the content of forage fibers. The coefficient of correlation between the digestibility and NDF, ADF, ADL and cellulose amounted to r=-0.84, r=-0.93, r=-0.91 and r=-0.86, respectively (Burritt et al., 1985). Very significant positive correlation was determined between the NDF content and content of ADF, ADL, hemicelluloses and cellulose amounted to (r=0.97, r=0.74, r=0.90, r=0.94). Significant positive correlation was determined between content of ADF and content of ADL and hemicellulose amounted to (r=0.66, r=0.77) and very significant positive correlation with cellulose content (r=0.99). Positive significant correlation was determined also between content of ADL and hemicellulose (r=0.76) and also between hemicellulose and cellulose content (r=0.72). Wermke (1986) established that there were maize hybrids with a low digestibility and a high content of stover cell wall constituents, as well as, there were hybrids with a high digestibility and a low content of cell wall constituents. These hybrids were defined as a dislocation type of hybrids. Hybrids with a high digestibility and a low NDF content of stover and a high NDF content of stover and a low ear digestibility are classified into types of hybrids with prolonged stover assimilation.

Table 3. Correlation dependence between digestibility, NFC and forage fibres of the whole plant of the ZP maize hybrids

	NFC	NDF	ADF	ADL	Hemicellulose	Cellulose
Digestibility	0.90**	-0.96**	-0.98**	-0.64*	-0.79**	-0.97**
NFC		-0.97**	-0.90**	-0.83**	-0.96**	-0.84**
NDF			0.97**	0.74**	0.90**	0.94**
ADF				0.66*	0.77*	0.99**
ADL					0.76**	0.55
Hemicellulose						0.72**
Cellulose						

The Figure 1 presents results of the digestibility of the whole ZP maize hybrid plant dry matter. The digestibility of the whole ZP maize hybrid plant dry matter ranged from 58.7% to 69.1%. The difference in the digestibility of the whole maize plant dry matter amounted to 10.4%. According to results obtained by Deinum et al. (1981) and Andreu et al. (1974) this difference varied from 2% to 3%, while Terzić (2006) and Terzić (2010) established the highest difference among observed hybrids of 11.52% and 8.56%, respectively. Differences in the digestibility of whole plant corn were statistically significant between the ZP hybrids.



$LSD_{0.05} = 1.07$
$LSD_{0.01} = 1.68$

Figure 1. Dry matter digestibility of the whole plant of the ZP maize hybrids

CONCLUSION

Obtained results showed that the NFC content in the whole maize plant of the observed different ZP hybrids varied from 33.20% to 41.22%. The difference in the NFC content of the tested ZP hybrids was 8.02%. The results also showed that the NDF, ADF, ADL, hemicellulose and cellulose contents in the whole maize plant of the observed different ZP hybrids varied from 42.57% to 50.84%, 19.32% to 25.74%, 1.63% to 2.51%, 23.26 to 26.07% and 17.69 to 23.62%, respectively. The digestibility of the whole ZP maize hybrid plant dry matter ranged from 58.7% to 69.1%. The difference in the digestibility of the dry matter of the whole plant between hybrids amounted to 10.4%. The differences in the contents of NFC and forage fibers affected the differences in digestibility of dry matter.

ACKNOWLEDGEMENTS

This study was financially supported by the Ministry of Education and Science of Republic of Serbia, Project TR31068.

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THE EFFECT OF BROKEN CORN INCLUSION IN BROILER NUTRITION ON CARCASS QUALITY

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ABSTRACT: In this paper the influence of broken corn and extruded broken corn addition in broiler diet on carcass quality was investigated. The experiment was conducted on broiler hybrid ROSS (n = 720) divided in three groups. The first group (control - C) was fed with commercial broiler feed mixture, while second and third groups were fed with commercial mixtures where 25% of corn was substituted with broken corn (experimental group E1) or extruded broken corn (experimental groups E2). Broiler fattening lasted 35 days, and food and water were provided ad libitum in the floor fattening system.
The obtained results indicate that the substitution of corn with broken and extruded broken corn did not affect significantly ($P > 0.05$) on the mass of cold carcasses. Also, shares of carcass main parts (breast, whole legs, back portion and tail end and wings) showed no significant ($P > 0.05$) differences between all three groups. The only significant difference ($P < 0.05$) was determined for mass of abdominal fat between control C (14.4 g) and experimental group E2 (21.2 g). Differences of meat yield in breast and whole leg of broilers from different groups were not registered as significant ($P > 0.05$). Based on the results obtained in this study it can be concluded that the use of 25% of broken or extruded broken corn in broiler diet did not have significant influence on carcass quality.
Key words: *broiler, feeding, broken corn, extruded corn, carcass quality*

INTRODUCTION

Poultry meat quality is a complex and multivariate property, which is affected by multiple interacting factors including genetics, feeding, husbandry, pre-slaughter handling, stunning and slaughter procedures, chilling processing and storage conditions. However it is likely that the effects exerted by ante-mortem handling (feed withdrawal, catching, crating, transport and lairage) and slaughter (hanging, stunning, killing, scalding, plucking, evisceration, chilling and processing) conditions on final product quality may be greater than those attributable to variation in husbandry practises. Many problems may occur at these stages that potentially increase the rate of mortality, carcass downgrading and meat quality.
Feeding strategy is the management factor which is most actively used as a quality control tool in the production of meat and in relation to improvement and/or control of performance, animal welfare, safety, nutritional value, and eating and technological quality (Andersen et al., 2005).
In the domestic production of forage mixtures, the corn has the leading position compared to other cereals, because of high energy contents (16.2 MJ/kg), starch, comparably big contents of oil and low level of cellulose (Bekrić, 1999). Proper thermal process provides the reduction of thermo labile antinutrients contents, to an acceptable level, and increases digestibility of some nutrients (protein, oil, carbohydrates), as well as the improvement of sensory features and the microbiological quality of the final product (Kormanjoš et al., 2007). Parallel with the reduction of antinutrients contents it is necessary to preserve nutritionally valuable thermo labile components, so the thermal process requires a compromise between the two efforts.
New technologies used to increase the nutritional value of feed are: toasting, hydrothermal refinement, micronisation, microwave treatment, bielectrical heat treating (Marsman et al.,

1998). In Serbia the most often used process are extrusion and hydrothermal process (Sakač et al., 2001; Filipović et al., 2008).
With 30% influence on carcass and meat quality (Rede and Petrović, 1997; Čepin and Čepon, 2001; Džinić, 2005; Džinić et al., 2009) feeding is considered to be the dominant premortal factor. Quality can be assessed both objectively and subjectively. Objective assessment tends to predominate throughout the supply chain prior to consumption by the consumer where quality attributes can be measured scientifically. Determination of carcass quality includes two main parts the definition of indicators for expressing the individual characteristics of quality and quantification of the inherent characteristics in relation to the overall quality.
The objective of this study was to determine the effect of broken corn and extruded broken corn addition in broiler diet on carcass quality.

MATERIAL AND METHODS

The experiment was carried on 720 broilers, hybrid ROSS. Broilers were divided in three groups, control group (C) and experimental (E1) and (E2) groups and fed under the same conditions in the period of 35 days. Experimental groups were fed with commercial mixtures where 25% of corn was substituted with broken corn (experimental group E1) or extruded broken corn (experimental groups E2). During whole broiler growing period water and feed were ad libitum. After growing and 12h starving period, 9 broilers from each group were slaughtered and processed by bloodletting, scalding, plucking and evisceration and chilled. Then chickens carcasses "ready to grill" from each group were cut in the basic anatomical parts: (SFRY 1/81 and 51/88). Cutting and deboning of breast and whole leg were applied in order to determine the breast whole leg meat yield quality. Analysis of variance (Duncan test) was used to test the hypothesis about differences among obtained results. The software package STATISTICA 8.0 (STATISTICA 8.0, 2008) was used for analysis.

RESULTS AND DISCUSSION

Examination of carcass quality (Table 1) of control and experimental groups shown that greater weight (1352,7 g) of cold carcass "ready to grill" was in experimental group E1 comparing to control (1286.2g), and experimental group E2(1298.5g). The difference between the chilled carcases of groups were not significant ($P > 0.05$). Measured masses of chilled carcasses „ready to grill“ were smaller than the results of Petričević et al. (2011) and Džinić et al. (2011) for female chickens of Ross 308 hybrid line, what could be attributed to the prolonged fattening period of 49 days. Greater breast weight (489.4 g) and whole leg weight (389g), the most important parts of the carcass, were found in chickens of experimental group E1 (fed with broken corn).The differences between the breast and whole leg weight of control and experimental groups E1 and E2 were not significant ($P > 0.05$). Average values of back portion and tail end were lower in chicken carcasses from control group comparing to the experimental groups. Average values of wings in carcasses of control and experimental groups were in the range of 139.5g (E2) to 148.4g (E1). Mass of abdominal fat was the lowest (14.4 g) in control group and significantly lower ($P<0.05$) comparing to chicken carcasses of experimental group E2 (21.2g). Mass of abdominal fat in chicken carcasses of experimental group E1 was numerically lower 16.2 g but not significant ($P > 0.05$) then in experimental group E2. The results are consistent with the results of Milosevic et al. (2007), who found significantly lower content of the abdominal fat in carcasses of broilers not fed extruded corn flower. According to Ljubojević et al. (2011) extruded corn influenced the reduction of abdominal fat contents in carcass of male and female Ross broiler but this difference was not statistically significant.

Table 1. The results obtained by weighting (g) the main parts of chilled carcasses of control and experimental chicken groups (mean values ± standard deviation)

Group	Chilled carcass mass^{ns} (g)	Breast mass^{ns} (g)	Mass of whole leg^{ns} (g)	Mass of back portion and tail end^{ns} (g)	Mass of wings^{ns} (g)	Mass of abdominal fat (g)
C	1286.2 ± 75.1	478.1 ± 37.6	376.2 ± 23.7	278.2 ± 16.2	141.5 ± 10.8	14.4^{a} ± 6.0
E1	1352.7 ± 97.5	489.4 ± 54.6	389.0 ± 26.3	297.9 ± 17.5	148.4 ± 10.3	16.2^{ab} ± 6.7
E2	1298.5 ± 63.0	468.2 ± 56.7	380.9 ± 22.9	287.2 ± 24.9	139.5 ± 11.8	21.2^{b} ± 5.3

Table 2. Breast meat, bones and skin weight (g) and share (%) in total breast mass of control and experimental chicken groups (mean values ± standard deviation)

Group	Breast (g)			Breast (%)		
	Meat^{ns}	Bones^{ns}	Skin^{ns}	Meat^{ns}	Bones^{ns}	Skin^{ns}
C	375.4 ± 37.9	64.8 ± 7.4	33.6 ± 4.9	78.4 ± 2.2	13.7 ± 2.3	7.0 ± 0.9
E1	383.4 ± 51.9	65.7 ± 9.1	35.1 ± 4.7	78.2 ± 2.9	13.5 ± 2.1	7.2 ± 1.2
E2	360.1 ± 62.2	68.7 ± 9.6	33.5 ± 4.1	76.5 ± 4.3	14.9 ± 3.3	7.2 ± 1.0

Chicken breast (Table 2) from experimental group E1 had higher (383.4 g) meat (65.7g) bones and (35.1g) skin weight in chilled carcass, but not significant ($P > 0.05$) in comparison with control and experimental group E2.Share of meat in breast within groups was in the range from 78.4% to 76.5% and differences between groups were not significant ($P > 0.05$).

Whole leg (Table 3) from experimental group E1 had the highest meat weight (265.0 g) and share of meat (68.1 %) in whole leg, while the meat weight (254.6g) had the lowest whole leg control group C. Share of meat (68.1 %) in the whole leg in group E1 was higher but not significantly ($P > 0.05$) compared to group E2 (66.9 %) and C (67.7%). Share of skin in the whole leg was the lowest (8.97%) in group C and significantly lower ($P < 0.05$) comparing to experimental group E1 (10.1 %) and group E2 (10.6%).

Table 3. Whole leg meat, bones and skin weight (g) and share (%) in total whole leg mass of control and experimental chicken groups (mean values ± standard deviation)

Group	Whole leg (g)			Whole leg (%)		
	Meat^{ns}	Bones^{ns}	Skin^{ns}	Meat^{ns}	Bones^{ns}	Skin
C	254.6 ± 18.2	82.2 ± 7.2	33.8 ± 3.2	67.7 ± 1.7	21.9 ± 1.7	8.97^{b} ± 0.6
E1	265.0 ± 20.3	79.6 ± 8.8	39.3 ± 6.4	68.1 ± 1.5	20.5 ± 1.9	10.1^{a} ± 1.5
E2	254.8 ± 17.1	80.5 ± 7.3	40.5 ± 4.9	66.9 ± 1.5	21.2 ± 1.7	10.6^{a} ± 1.0

The results obtained in Table 4 showed that experimental group E1 had the highest breast meat, whole leg meat and total meat weight in chilled carcass comparing to the control and experimental group E2. Share of breast meat, whole leg meat and total meat in chilled carcass, between control and experimental groups were not significant ($P > 0.05$).

Table 4. Breast meat, whole leg meat and total meat weight (g) and share (%) in chilled carcass of control and experimental chicken groups (mean values ± standard deviation)

Group	Meat (g)			Meat (%)		
	Breast^{ns}	Whole leg^{ns}	Total^{ns}	Breast^{ns}	Whole leg^{ns}	Total^{ns}
C	375.4 ± 37.9	254.6 ± 18.2	629,9 ± 49,9	29.1	19.7	48.9
E1	383.4 ± 51.9	265.0 ± 20.3	648.4 ± 64.1	28.3	19.6	47.9
E2	360.1 ± 62.2	254.8 ± 17.1	614.9 ± 55.9	27.7	19.6	47.3

CONCLUSIONS

The diet with extruded corn did not affect significantly (P > 0.05) on mass of chilled carcass. Quality resulted in increased weight of carcass, higher share of breast meat in chilled carcass, higher protein and reduced free fat content in breast meat (P > 0.05), comparing to control group. Shares of carcass main parts (breast, whole legs, back portion and tail end and wings) showed numerically (P > 0.05) differences between control and experimental groups. Significant difference (P < 0.05) was determined for mass of abdominal fat between control C (14.4 g) and experimental group E2 (21.2 g). Experimental group E1 had the highest breast meat, whole leg meat and total meat (P > 0.05) weight in chilled carcass comparing to the control and experimental group E2.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project III046012 financed by the Ministry of Education and Science, Republic of Serbia.

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DEVELOPMENT OF A HEME IRON FEED SUPPLEMENT FOR PREVENTION AND THERAPY OF ANEMIA IN DOMESTIC ANIMALS

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ABSTRACT: Animal blood produced in slaughterhouses as a by-product of the meat industry represents serious biohazard. Transformation of wasted slaughterhouse blood to the highly valuable product(s) may partially solve the problem of disposing slaughterhouse blood wastes and at the same time, isolated heme concentrates can be used to fortify feed. The aim of this study was to optimize the isolation process of bovine hemoglobin from slaughterhouse blood by gradual hemolysis in a membrane bioreactor. 35 mM Na-phosphate/NaCl buffer solution of pH 7.2-7.4 was identified as the optimal external medium providing effective gradual osmotic hemolysis with an extent of hemolysis of 88%. The hemoglobin purity of >80% was confirmed by SDS-PAGE. Kinetic studies showed that maximal concentration of hemoglobin was reached after 40 min, but the process cycle at which recovery of 83% was achieved, lasted for 90 min. Methemoglobin levels remained below 2% with hemoglobin concentration of 4.8 ±0.5 gL^{-1} at the end of process. In order to produce ready-to-use feed additive for prevention of iron deficiency anemia in domestic animals, future studies should be oriented toward development of down-stream processes, such as tangential flow filtration and lyophilization.
Key words: *slaughterhouse blood, bovine hemoglobin, gradual hemolysis, membrane bioreactor*

INTRODUCTION

Iron deficiency is the most common and widespread nutritional disorder in the world. As well as affecting a large number of children and women in developing countries, it is the only nutrient deficiency which is also significantly prevalent in industrialized countries (http://www.who.int/nutrition/topics/ida/en/index.html). Besides, as the main cause of hypochromic anemia, iron deficiency represents a major problem in several segments of the livestock industry, whereas piglet anemia is the most wide-spread.

Food fortification is generally considered to be the best long-term strategy to combat iron deficiency (Cook and Reusser, 1983). Most of the supplements (for human or veterinary use) for prevention and treatment of hypochromic anemia existing on the market are based on non-heme iron (inorganic and organic iron salts). Another source of iron is heme iron, which is only found in myoglobin or hemoglobin. Compared to non-heme iron, the bioavailability of iron from heme iron sources may be 2- to 7-fold higher than that of iron from non-heme sources (Hurrell 1997; Seligman et al.; 2000; Quintero et al. 2008). Also it has shown that absorption of heme-iron is not dependent upon dietary composition (Conrad et al., 1966) and produces little side effects (Frykman et al., 1994).

In animal blood, hemoglobin accounts for more than half of the blood proteins and 90% of the cell fraction proteins. Animal blood produced in slaughterhouses represents the most problematic by-product of the meat industry due to the high volumes generated and very high pollutant load of this biohazard. At the same time it represents an inexpensive source of heme-iron. Transformation of one wasted slaughterhouse blood to the highly valuable product, such as hemoglobin may partially solve the problem of disposing slaughterhouse blood wastes, especially for developing countries. Hemoglobin has already been used with success for food fortification (Hertrampf et al., 1990; Walter et al., 1993). Its low iron content (0.35%) limits whole hemoglobin use as a food supplement in treatment of iron deficiency in humans, but it stands for suitable compound of common bulky feed for domestic animals.

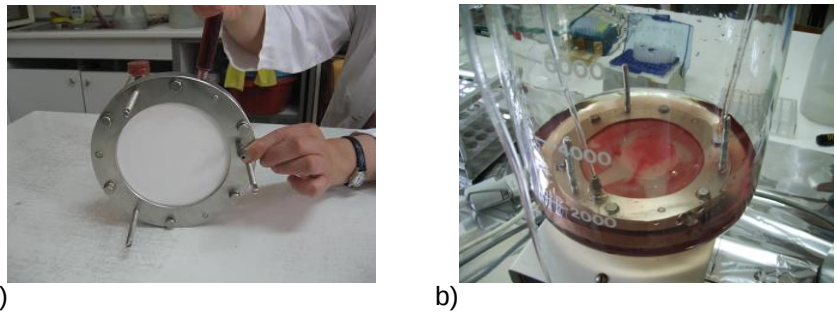
The isolation and purification of hemoglobin is the essential and first step in the heme iron production process. The aim of the investigation presented in this paper was to optimize the isolation process of bovine hemoglobin by gradual hemolysis in the originally designed membrane bioreactor (Bugarski et al., 2003), according to the osmotic properties of erythrocytes from bovine slaughterhouse blood. The process of gradual osmotic hemolysis is discussed in terms of extent of hemolysis, erythrocyte volume change, the time course of hemoglobin release, and morphological changes in red cells associated with osmotic swelling.

MATERIAL AND METHODS

Bovine blood (Slaughterhouse "Ambar", Surčin, Serbia) was taken from jugular vein of Holstein-Friesian calves and collected in a glass bottle containing 3.8% Na-citrate as an anticoagulant agent. Whole blood was centrifuged (2100 × g) at room temperature, and plasma proteins and leucocytes (buffy coat) were removed by vacuum aspiration. The three step washing with isotonic (0.9% w/v) saline solutions (Hemofarm, Vršac, Serbia), was sufficient to remove contaminating plasma proteins according to Andrade et al. (2004). After washing, erythrocytes were resuspended in isotonic (0.9% w/v) saline solutions at 40% of hematocrit. The cyanmethemoglobin method (Van Assendelft et al., 1984) was used for determination of hemoglobin concentration in erythrocyte suspension. The concentration of methemoglobin was determined by the method described by Palmer et al. (2008). Hematocrit was measured by the microhematocrit method (Sabine and Nickolai, 1952). Erythrocytes were counted on a Spenser hemocytometer. Osmotic fragility of bovine erythrocytes was determined by the method of Beutler (1983). The concentration of NaCl producing 50% of hemolysis (H_{50}) was calculated by the GraphPad Prism 3.0 software program. The osmotic swelling index and extent of hemolysis were determined according to methodology by Vitvitsky et al. (2000).

Membrane bioreactor

The main part of the membrane bioreactor is the module which was designed and constructed at the Department of Chemical Engineering, Faculty of Technology and Metallurgy, University of Belgrade, Serbia. It is composed of three stains-less still ring-like plates with an outside diameter of 16.6 cm and inside diameter of 11.5 cm, and two cellulose acetate membrane filters with 0.8 µm pores (Sartorius AG, Goettingen, Germany) placed between the rings (Fig. 1). The module has inside volume of 104 mL, while the total volume is 290 mL. The module is charged through an orifice placed at a lateral side of the device and closed with stainless still bolt. The module was placed in a 10 L glass vessel with an internal diameter of 22 cm.



a) b)

Figure 1. Membrane reactor: a) charging of module b) process of hemolysis

Gradual hemolysis in the membrane bioreactor

The module was charged with 100 mL of erythrocyte suspension (40% of hematocrit) and placed in the reactor vessel previously filled with 950 mL of isotonic saline solution. Hemolysis was performed by pumping hypotonic buffer solution at a flow rate of 900 mL/h using a peristaltic pump (IP610 Beomedicina, Belgrade, Serbia). The bioreactor was placed on an orbital shaker (Yellow line OS 5 basik (Ika Werbe GMBH & Co, Germany)) for continuous mixing at 320 rpm. Hemoglobin released from erythrocyte membranes diffused throughout the module, and accumulated in the external solution. The release of hemoglobin was followed by measuring optical density at 540 nm (OD_{540}) of the external solution by using UltroSpec 3300prp spectrophotometer (AmershamBioscience, Uppsala, Sweden); the aliquots of the external solution were withdrawn in 1 min time intervals for the first 15 min of hemolysis, in 3 min time intervals for the next 15 min, in time intervals of 5 min for the next 30 min and in the time intervals of 10 min by the end of the experiment. The samples were centrifuged and OD_{540} of the supernatant was measured as described above. The hemolytic process was stopped after 90 min.

The concentration of hemoglobin in hemolysates was determined by the cyanmethemoglobin method (Van Assendelft et al., 1984). Protein concentration in the suspension of erythrocyte ghosts was measured using BCA Protein Assay Kit (Pierce Biotechnology, IL, USA). Efficiency of the process was assessed in terms of recovery rates for hemoglobin (RR%).

The recovery rate was calculated according to the following equation:

$$RR\% = \frac{m_h}{m_i} \times 100\% \quad (1)$$

where m_i is the initial mass of hemoglobin in an erythrocyte suspension prior to hemolysis and m_h is the mass of hemoglobin in a hemolysate at the end of the process.

SDS electrophoresis

SDS polyacrylamide gel electrophoresis of hemolysate, obtained by gradual hemolysis (40 μg of hemoglobin) was carried out in SE 260 Mighty Small II Vertical Slab Electrophoresis Unit (GE HealthCare LifeScience, USA) in 12% gel according to the procedure of Laemmli (1970). Gels were stained in Coomassie blue R-250 and distained in an acetic-acid/methyl alcohol aqueous solution. Densitometric analysis was performed using ImageMaster TotalLab v1.1 software (GE HealthCare LifeScience, USA). PageRuler Prestained Protein Ladder (Fermentas) molecular size markers, with apparent molecular weight of 170, 130, 95, 72, 55, 43, 34, 26, 17, and 10 kDa, were used.

RESULTS AND DISCUSSION

Although the process of gradual osmotic hemolysis in the membrane bioreactor is already developed for isolation of human hemoglobin (Bugarski et al., 2003), it was necessary to adapt the process according to osmotic characteristics of bovine erythrocytes.

Since many properties (such as cell diameter, volume and shape, membrane lipid composition, ionic composition, etc.) differ between human and bovine erythrocytes which may affect the osmotic properties of erythrocytes, the first aim of this study was to investigate osmotic fragility (OF) of bovine erythrocytes. Fig. 2 represent mean cumulative and derivative bovine erythrocyte osmotic fragility curves drawn from mean values for three experiments. Herein we found the H_{50} value of 106 ± 9 mM for Holstein-Friesian calves investigated in our study. Also, results showed that osmotic fragility of bovine erythrocytes from slaughterhouse blood taken with sodium citrate as an anticoagulant remained unaltered four days. Derivative curve revealed that bovine erythrocytes were lysed in buffer with molarity lower then 70 mM.

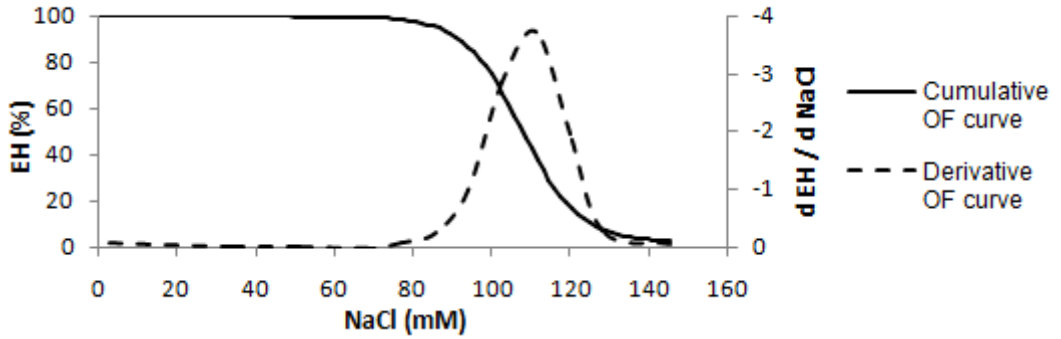


Figure 2. Mean cumulative and derivative osmotic fragility curves of bovine erythrocyte drawn from values for three experiments

The next step was to define the optimal concentration of Na-phosphate / NaCl solution at which gradual hemolysis will occur, giving hemoglobin in a high yield and preventing the rupture of cell membranes. Fig.3 demonstrates kinetics of changes in swelling index and extent of hemolysis induced in bovine blood samples by buffer solutions of decreasing molarities. We have defined the term "swelling index" equal to the relative volume change of erythrocytes. It is the difference between the hemolytic and isotonic volumes of a cell expressed as a relative value to the initial i.e. isotonic volume. A decrease in buffer molarities caused changes in the intracellular ion concentrations and an increase in cell volume and, consequently, decrease in the suspension hematocrit value; this was accompanied by appreciable hemolysis.

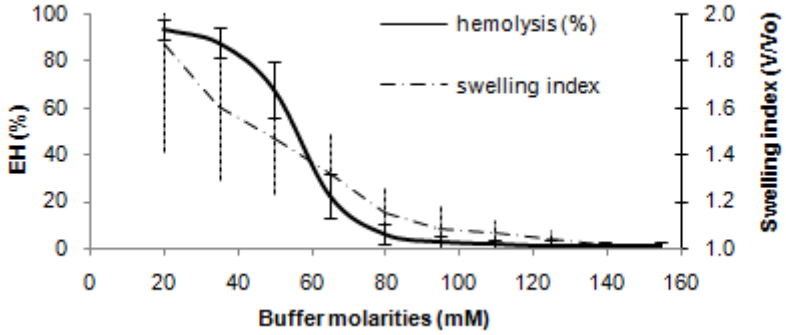


Figure 3. Extent of hemolysis and swelling index of bovine erythrocyte suspension as a function of Na-phosphate / NaCl buffer molarity. Results present mean ± SD values of five experiments.

The results show that ~90% of cells hemolyzed at ~35 mM solution. A 35 mM solution, caused a swelling of 1.84. on the average. With 35 mM solution, the residual hemoglobin in suspension of erythrocyte ghosts at the end of the process was 0.052 ± 0.002 (expressed as ratio of OD_{540} and hematocrit). For a sake of comparison, the same parameter was significantly higher (0.084 ± 0.016) after treatment of erythrocytes with 20 mM buffer solution. Considering all the facts named here 35 mM Na-phosphate / NaCl buffer pH 7.2-7.4 solution was identified as the optimal external medium permitting gradual osmotic hemolysis and isolation of hemoglobin in a high yield.

Further on, gradual hemolysis was performed in the membrane bioreactor with 35 mM Na-phosphate / NaCl buffer under a flow rate of 900 mL/h, during 90 min. The release of hemoglobin was followed by measuring OD_{540} of the external solution (Fig. 4). Maximal concentration of hemoglobin in the surrounding solution was reached after 40 min. The time interval needed to attain recovery rate of hemoglobin more than 80 % was 90 min and this period was set as duration of one process cycle in the membrane bioreactor. The concentration of hemoglobin at the end of the process cycle was 4.8±0.5 gL^{-1}.

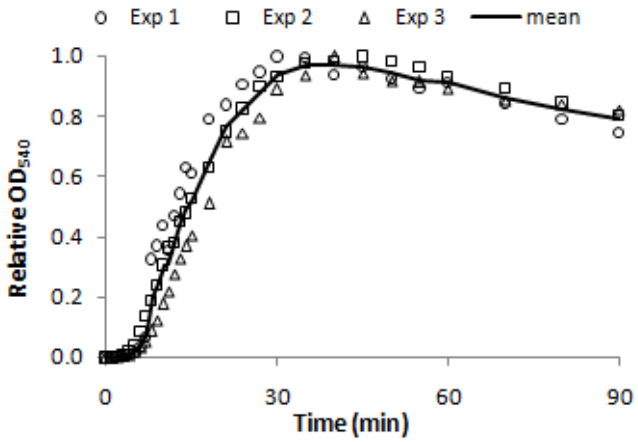


Figure 4. The time course of hemoglobin release during gradual hemolysis of bovine erythrocytes in the membrane reactor system. Results present three different experiments and its mean value.

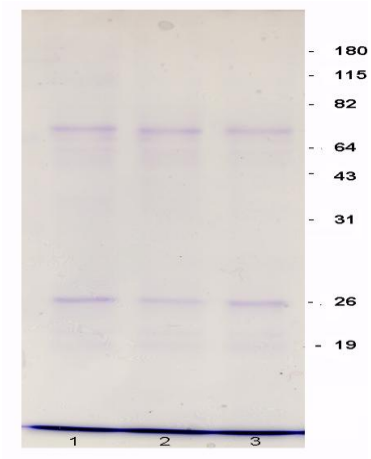


Figure 5. SDS-PAGE electrophoresis analyses for hemolysate samples obtained in three independent hemolysis cycles (Lines 1, 2 and 3)

The optimized process provided high yields of hemoglobin. For three experiments, the mean recovery rate of hemoglobin was 85 ± 11%. The fraction of methemoglobin was less than 2%. Absorption spectra of the isolated bovine hemoglobin from 250-800 nm confirm that isolation process yielded predominantly to oxyhemoglobin (figure not shown). The purity of hemoglobin solution was analyzed by SDS-PAGE (Fig. 5). Densitometric analysis showed that protein fraction which corresponds to globin chains (16 kDa) represented 84.90 ± 0.64% of the protein content. After process of gradual hemolysis is finished, empty, intact erythrocyte ghosts reside in the reactor chamber, while hemoglobin solution was separated by the membranes. Therefore, no additional steps were needed to collect lipid impurities. This is another advantage of the hypotonic dialysis in the membrane bioreactor as compared to most of isolation procedures described in literature which comprise methods like high speed centrifugation or filtration through glass wool for removing cell debris and membrane phospholipids (Sun and Palmer, 2008).

CONCLUSION

As a result of the optimized process of gradual hemolysis in membrane reactor described in this paper, pure bovine hemoglobin solution has been obtained from wasted slaughterhouse blood. With the aim to produce a dry powder from the obtained hemoglobin solution, which

could be used as the heme concentrate source in the fortification of feed, downstream processes such as tangential flow filtration and lyophilization merit further investigation.

ACKNOWLEDGEMENT

This research was funded by grants III46010 and E!4486 from the Ministry of Education and Science, Government of the Republic of Serbia

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PHYTASE AS ADDITIVE TO ANIMAL DIET AND ITS INFLUENCE TO DRESSING TRAITS OF MEAT

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ABSTRACT: The aim of examination in this work was: scientific determination of data about influence of phytase added into diets for fattening chickens, by lower level of mineral source of phosphorus to the finishing body weights had to some slaughtering traits of meat. Experimental examinations were done through the experiment of feeding fattening chickens of Arbor Acres strain. In the experiment were 220 chickens, divided into two groups, considering the different formulation of diets. K-control group (110 chickens), as the source of phoshorus gained by diet dicalcium-phosphate (DKF) at level 2%, O-I group gained by diet DKF-1% with addition of 0,1% enzyme of phytase. Standard fattening technology was applied in lasting period of 42 days. At the end of experiment, 14 head of animals were sacrificed (7 male and 7 female) out of each group. Necessory measuring were done: body weights before slaughtering; weight of dressed warm carcass; weight of the basic parts of carcass. Dressing percentages were calculated as well as yield and share of the basic parts considering dressed carcasses. Gained results show that the chickens of O-I group (DKF-1%+0,1% of phytase) gained better finishing body weights (2006,50gr) in comparison to K-group (DKF-2%)-1875,63gr ($P<0,05$). Also O-I group had better dressing percentages (65,66%), K-group (63,16%) ($P<0,05$). Further, yield of meat of the first category, by O-I group (771,56gr) and K-group (694,79gr) ($P<0,05$).
Key words: *chickens, mineral sources of phosphorus, phytase, production, meat quality*

INTRODUCTION

Application of mineral sources of phosphorus in animal diets takes risk to health of animal and human race. They are qualified as great canse of environmetal pollution (Otto,1998; Kies et al. 2001). They also have significant influence to the price on animal products, because they are expensive. Besides that, lately the new approach was established considering application of mineral sources of phosphorus. The ail of that is decreasing or complete leaving it out of animal diets. In these kinds of diets formulation, enzyme phytase has been added which with its hydrolisis effects, makes available phytinic sources of phosphorus from herbs diets for monogastric animals and poultry (Harter-Dennis, 2000). Diets which have additional phytase and have been used for feeding fattening chickens have positive influence to production traits and improvement of meat and bones quality.
The aim of these examinatins was to complete scientifical determination of data about the influence of phytase added into the diets for fattening chickens by lower level of mineral source of phosphorus to the finishing body weights and dressed carcass traits of meat.

MATERIAL AND METHODS

The experimental examinations were done while the period of feeding fattening chickens of Arbor Acres strain was in progress. There were 220 chickens in the experiment, which were divided into two groups: K-control (110 chickens) and the experimental group (110 chickens). The demand of the experiment was to divide chickens into two boxes considering the different source formulation of phosphorus. During the experimental period K-group gained per diet dicalcium-phosphate (DKF) at level of 0,2%, while O-I group gained per diet 0,1% with addition of 0,1% phytase. The fattening chickens were fed with the compositon made of same raw materials.

The experiment of feeding has lasted for the period 42 days. At the end of the experiment, 14 chickens from each group were sacrificed (7 male and 7 female). The body weight, weight of hot dressed carcass as well as the weight of the basic parts of dressed carcass were measured before the slaughtering.
Dressing percentages were calculated as well as yield and share of the basic parts in dressed carcass (per each chicken) – (measuring was done with precision balance ±0,1gr.

RESULTS AND DISCUSSION

Considering that the yield of dressed carcass, dressing percentages as well as yield and share of some meat categories of dressed chickens carcass, very important fact of quality, influence of food tretments to the mentioned traits was also observed.
The results of examinations (table 1 and 2, figure 1 and 2) show that: the chickens of O-group (1% DKF+0,1% phytase) gained better average finishing body weights (2006,5g) in comparison to K-group (2% DKF) (1875,63g) (P<0,05). Also, O-group had better percentages (65,66%) K-group (63,16%) (P<0,05). Further, yield of meat of the first category, by O-I group (771,56gr) and K-group (694,79gr) (P<0,05).

Table 1. Yield of dressed carcass of the chickens from examined groups (n=14)

Grupa		Body weight before slaughtering (g)	Dressed carcass		Cold carcass		Loss during cold	
			Weight (g)	Dressing percentage %	Weight (g)	Dressing percentage %	Weight (g)	Dressing percentage %
K	$\bar{x}$	1878,93	1187,5	63,16	1176,07	62,53	11,78	1,05
	Sd	146,87	108,66	1,97	108,17	1,93		
	CV	7,82	9,15	3,13	9,20	3,08		
	ind.	100	100	100	100	100	100	100
0-I	$\bar{x}$	1964,28	1290,0	65,66	1278,21	65,06	11,78	0,92
	Sd	120,87	100,29	2,92	99,82	2,93		
	CV	6,15	7,77	4,45	7,81	4,50		
	ind.	104,54 +4,54	108,63 +8,63		108,68 +8,68		100 -	
		*P < 0,05	*P<0,05		*P<0,05			

Table 2. Yield and share of some meat categories of dressed carcasses of chickens (n=14)

Parameter	Group	Weight of cold carcass,gr	The first category	
			gr	%
Average	K	1176,07	694,79	59,09
Sd		108,17	67,70	
Cv		9,20	9,74	
Index		100	100	
Average	O-I	1278,21	771,56	60,40
Sd		99,82	59,66	
Cv		7,81	7,73	
Index		108,68	111,05	
Diference		+8,68	+11,05	
			*P<0,05	

As for as the finishing body weights of the chickens, significant differences among groups were observed, considering the group which gained by diet DKF (K) and the group which gained lower level of DKF with addition of phytase 0,1% (O-I group). This difference in finishing body weights had direct influence to the dressing percentages, and also to the yield the basic parts of carcass (breast, drumstick, thigh). These results are in comrehension with the results of many authors, who clainred that the different variation diets formulations, could increase adsorption of phytinic phosphorus and other digestable consist of feeding mixtures (Sebastian et al. 1998, Radović i sar. 2011).

CONCLUSIONS

Considering the results of the examination gained by the experiment of feeding fattening chickens, we came to the conclusion that: addition of phytase into diets for fattening chickens, at the lower level of mineral source of phosphorus, had the positive effect to the observed traits in our examination. So that means, that phytase had its influence to the improvement of production and slaughtering traits of chickens. Further on, diets with phytase, fulfill ecological demands, considering decrasing of environmental pollution.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project 046012 financed by the Ministry of Education and Science, Republic of Serbia.

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EFFECT OF THE HIGH POLYUNSATURATED FATTY ACIDS LAYER DIETS SUPPLEMENTED WITH ANTIOXIDANTS ON THE CHOLESTEROL LEVEL OF EGG

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ABSTRACT: The paper presents the results of a trial conducted for 8 weeks on 90 Lohmann Brown layers (43-51 weeks of layer age) assigned to 3 groups (3 layers per cage). The control group (C) received a basal corn (53.35%), corn gluten (1%) and soybean meal (26%) diet. The diets for the experimental groups (E1 and E2) has the same basal diet as C group but included flax seeds (7%), buckthorn oil (1%) and antioxidants, as follows: 250 ppm vitamin E (E1), and 250 ml/kg marigold extract (E2). Feed intake and egg production were monitored throughout the experiment. Egg samples (15 eggs/group) were collected on weeks 2, 5 and 8. Average samples (3 eggs/sample) were formed from the collected eggs for each variant and assayed for the gross chemical composition and yolk cholesterol. There are different results ($p \leq 0.05$) regarding feed intake (105,616±1,085 g) for group E1 compared with C group (111,198±1,037g) and E2 (110,769±0.924). Also, are different results ($p \leq 0.05$) regarding the egg weight (63,109±0,527 g) for group E1 compared with C group (62,027±0,209037g) end E2 (61,744±0,104 g). The yolk cholesterol decreased throughout the experimental period (43-51 weeks of layer age) in all groups, which shows that this fact was not related to the diet. Thus, the decrease was 18.27% for C group (0.559 ± 0.13 g col/fresh yolk in the end of the experiment, compared to 0.684 ± 0.048 g col / fresh yolk, initial value), 7.15% for E1 (0.519 ± 0.053 g col / fresh yolk in the end of the experiment, compared to 0.559 ± 0.07 g col/fresh yolk, the initial value) and 8.45 % for E2 (0.520 ± 0.034 g col / fresh yolk in the end of the experiment, compared to 0.568 ± 0.042 g col/fresh yolk, initial value).
Key words: *layers, cholesterol, marigold extract, antioxidants, egg*

INTRODUCTION

The increasing concerns for human health, its relation with the structure and quality of animal feeds and the association of trace dietary ingredients (essential fatty acids, vitamins and minerals) to positive effects on human health, explain the drive of nutritionists towards finding rich sources of such nutrients. The hen egg is a basic natural food for humans. The egg has large amounts of protein (it has all the 9 essential amino acids) and other essential nutrients such as polyunsaturated fatty acids, vitamins, minerals etc. The only worrying issue for the consumers is the cholesterol level. Many physicians consider that the egg is the main source of dietary cholesterol and therefore the decrease of yolk cholesterol is a subject of top interest for layer feeding. Presently, there is plenty of documentation proving that the fatty acids profile can be changed by feeding, decreasing the ratio of ω:6 to ω:3 fatty acids as well as the ratio of the saturated to unsaturated fatty acids. The use of feeds rich in ω:3 fatty acids in layer feeding increases directly proportional the concentration of these acids in the yolk. These eggs rich in:3 fatty acids are alternatives to fish or to other foods from oleaginous seeds, also rich in ω:3. The change of the fatty acids profile and the decrease of the cholesterol level is done almost entirely through feeding. The specific feeding way is to enrich layer diets in omega 3 polyunsaturated fatty acids (Alvarez et al., 2005). Besides the addition of raw ingredients rich in fatty acids with double bonds, the nutritionists must also provide the proper amounts of antioxidants which will remove the unpleasant effects of self-

oxidation (rancidity) of the dietary lipids. The first signs are the unpleasant smell and the spoiled taste. Therefore, it is very important that the modification of the dietary composition doesn't affect the quality and the functional properties of the eggs. Vitamin E is the most often used antioxidant which is added to the compound feeds through the vitamin premix. Monounsaturated and polyunsaturated fatty acids were patented for their capacity to lower egg cholesterol. The decreased of yolk cholesterol is preceded by the decrease of the serum cholesterol, which is precursor of yolk cholesterol. Other methods to decrease the yolk cholesterol include the use of copper and chrome (Balevi and Coskun, 2004; Guruprasad et al, 2006). Recently, several teams of researchers focused on the use of phytoadditives to reduce the cholesterol level. Akhtar et al. (2003) used Nigella sativa seeds in layer diets, which inhibit cholesterol synthesis and stimulate the secretion of bile acids, both processes decreasing egg cholesterol.
The purpose of the paper was to study the effect of the diets rich in polyunsaturated fatty acids supplemented with (natural or synthetic) antioxidants on the cholesterol level of the hen eggs.

MATERIAL AND METHODS

The experiment was conducted on 90 Lohmann Brown laying hens (43-51 weeks) for 8 weeks. Birds were grouped taking into consideration body weight, being divided into 3 groups (30 birds / group) and housed in cages structured on three levels, according with modern and in accord with European standards. The structure of cages has allowed the registration of daily intake and excreta. Batteries were placed in a hall whose ambient temperature was 25°C during the experiment. Incandescent lighting throughout the experimental period was performed by a scheme with up to 16 hours daily light. Food and water were administrated *ad libitum*. During all 8 weeks, all experimental parameters were ensured on the specific microclimate according to growth technology and age of birds. The control group (C), it was used a ratio based on corn, corn gluten, soybean meal and raw materials rich in polyunsaturated fatty acids (PUFA) - flaxseed (7%), sea buckthorn oil (1%). Experimental groups diets (E1 and E2) differed from control by using two antioxidants, a synthetic antioxidant (vitamin E) and a natural antioxidant (marigold extract) (Table 1). Group E1 diet received the antioxidant supplement of 250 mg / kg vitamin E, and group E2 diet received 250 ml natural antioxidant extract of marigold / 100 kg CN. In terms of energy-protein, all three structures were isocaloric and izoproteic diets (Table 1). During the experiment, daily, were monitorized and recorded production parameters on feed consumption, and productivity of laying and eggs weight. Every two weeks samples were taken from eggs (15 eggs / serial / group). From collected eggs, medium samples were formed (3 eggs / sample) for each version in order to determine the gross chemical composition and cholesterol content in egg yolk. Physical parameters of eggs parameters (egg weight, yolk color index and the degree of freshness Haugh) were measured using the device: Egg Analyzer (Sanovo Engineering A / S, Denmark). Feed samples were taken at the beginning of the experiment, and were analyzed for nutrients content; dry matter, crude protein, fat, crude fiber and ash were estimated according to the ISO methods (The Romanian Standardized Association (ASRO)). For fatty acid analysis, and cholesterol from yolk, were extracted by the methanol-hexane procedure. The samples were analyzed by using a Perkin Elmer gas chromatograph (Clarus 500, USA). One way ANOVA analysis was performed to investigate the statistical differences between groups for all parameters analysed. Further differences between means were determined by the least square difference Fisher procedure. Values of $P< 0.05$ were considered significant.

Table 1. Ingredient of the feed mixture, %

Specificatie	C	E1	E2
Corn	53.35	53.35	53.35
Soybean meal	26	26	26
Gluten	1	1	1
Sea buckthorn oil	-	-	1
Flaxseed	7	7	7
Marigold Extract	-	-	0.25
Monocalcium phosphate	1.25	1.25	0.25
Calcium carbonate	8.9	8.9	8.9
Salt	0.3	0.3	0.3
Methionine	0.15	0.15	0.15
Choline	0.05	0.05	0.05
Premix	1	1 (P2 –with vitamin E)	1
TOTAL	**100**	**100**	**100**

RESULTS AND DISCUSSION

In Table 2 zootechnical performances are presented throughout the experimental period. There were significant differences ($P \leq 0.05$) on average daily consumption recorded, a 5.01% decrease from E1 compare to group C and 4.6% compare to group E2. Instead, the group E1 egg weight was significantly ($P \leq 0.05$) compared with the other two groups (C and E2). As expected, the lowest feed efficiency was registered in group E1 (1.961 kg CN / kg egg), differentiated significantly ($P \leq 0.05$) by E2 group. Laying intensity was 6.12% higher in group C compared to E1 and 4.33% higher than E2 (Table 2), but was not affected.

Table 2. Performances

Specification	C	E 1	E2
Average daily intake, g/zi	111,198±1,037 ^{b}	105,616±1,085 ^{a,c}	110,769±0,924 ^{b}
Feed efficiency, kgNC/kg ou	1,972±0,028 ^{c}	1,961±0,028 ^{c}	2,07±0,033 ^{a,b}
Laying intensity, %	91,63±1,103 ^{b,c}	86,021±0,86 ^{a}	87,661±1,205 ^{a}
Egg weight/period, g	62,027±0,209 ^{b}	63,109±0,527 ^{a,c}	61,744±0,104 ^{b}

Note: a = significantly diffrent ($p \leq 0,05$) compare to C ; b = significantly diffrent ($p \leq 0,05$) compare to E1; c = significantly diffrent ($p \leq 0,05$) compare to E 2;

Figure 1 presents egg components weights. Eggs collected from experimental groups had a higher weight, but insignificant (p> 0.5) from group C.

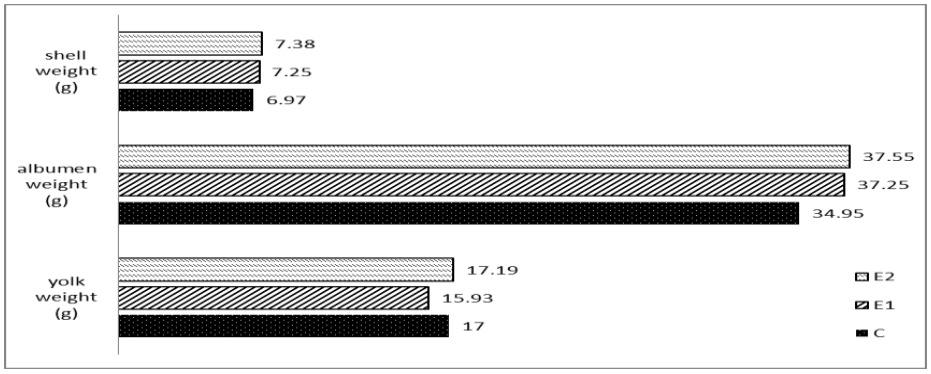

Figure 1. Egg components weights throughout the experiment

Due to the use of feed sources rich in fat, a major requirement is suggested concerning for feed quality while temporally preserving and consequently the eggs obtained during this experiment. To assess the temporally feed quality keeping, feed samples were taken from which were determined indices of fat degradation: acidity, peroxide index KREISS reaction (for fats oxidation evaluation), initially, at 14 days and 28 days. Evolution of the peroxide (Table 3) shows that at 28 days in group C appeared the phenomenon of degradation of fat. Confirmed, also, by Kreiss reaction (positive). Acidity, also, was consistently higher in this group, due to the insufficient level of vitamin E in the ration. The two experimental groups recorded lower values in terms of fat acidity index and peroxide. The results presented in Table 3 show that using a high-fat feed rations is strongly conditioned by the inclusion of antioxidant sources. For experimental groups, was given both the antioxidant effect of vitamin E supplementation (intake group E1) and the extract of marigold (intake group E2).

Table 3. Evolution of odder fat indices degradation

Specification	C	E 1	E2
Peroxid index (ml thiosulfat 0.1 N/g Gr)			
- initial	1.33	1.13	1.04
- 14 days from manufacturing	1.51	1.36	1.24
- 28 days from manufacturing	1.85	1.22	1.63
Fat acidity (mg KOH / g grasime)			
- initial	27.14	15.08	13.24
- 14 days from manufacturing	29.15	17.43	16.52
- 28 days from manufacturing	31.73	19.82	17.18
KREISS reaction			
- initial	Negative	Negative	Negative
- 14 days from manufacturing	Suspicious	Negative	Negative
- 28 days from manufacturing	Pozitive	Positive	Pozitive

During the experiment 15 eggs / group were collected randomly (at weeks 0, 2, 4, 6 and 8) of which medium samples were formed to establish physical and chemical parameters of egg yolk (Table 4). Egg samples collected were analyzed chemically, on the levels of protein, fat, ash and dry, and content in fatty acids, vitamin E and cholesterol.

Table 4. Egg yolk physica parameters (average values / group)

Specification	C	E1	E2
pH-whites	8,995±0,008	9±0,008	9,002±0,008
pH-yolk	5,998±0,006	6,008±0,005	6,008±0,005
Colour intensity	5,9±0,233	5,909±0,251	5,692±0,208
Vitamin E, mg%yolk	1,677±0,75 $^{b.c}$	4,757±0,164 ^{a,c}	1,075±0,105 ^{a,b}
H units	70,5±2,525	65,836±3,968	64,969±2,495

Note: a = significantly diffrent ($p \leq 0,05$) compare to C ; b = significantly diffrent ($p \leq 0,05$) compare to E1; c = significantly diffrent ($p \leq 0,05$) compare to E 2;

In Table 4, the physical parameters of egg yolk are presented. There were no significant differences between groups for pH whites values, pH yolk values and yolk color intensity. In terms of vitamin E concentration in egg yolk, the data presented previously an increase in concentration of vitamin E in group E1. This is due to increasing dose of vitamin E / kg CN group E1. Out of the total fatty acids determined in egg yolk, in Figure 2, changes are presented regarding concentrations of polyunsaturated fatty acids: linoleic (ω: 6) and linolenic (ω: 3) which are of major importance for the body. (Calvani and Benatti, 2003, Barlow and Pike, 1991; Mantzioris, 2000; Simopoulos, 2000; Kralik et al.(2005);

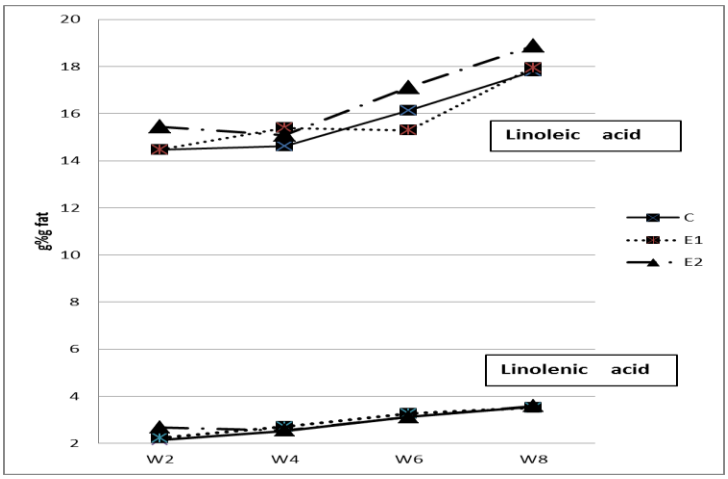

Figure 2. Temporally evolution of polyunsaturated fatty acids (linoleic and linolenic)

Figure 2 shows the values determined for the two presented acids ($p \leq 0.05$) higher at the end of the experiment compare to the beginning of the experiment for all three groups studied. Concentrations obtained in eight experimental weeks were significantly higher ($p \leq 0.05$) for group E2 compare to C for linoleic acid. For linolenic acid, there were significant differences between groups at the end of the experiment. Polyunsaturated fatty acid concentration was significantly higher in group received additional marigold, compared to group C. In terms of polyunsaturated fatty acids ratio ω 3 / ω: 6, values were comparable for the three studied groups.

Table 5. Ratio of mono-and polyunsaturated fatty acids in egg yolk

Specification	C	E 1	E2
PUFA	25,09	25,56	26,60
SFA/UFA	0,47	0,49	0,47
PUFA			
ω:3 / ω:6	0,180	0,177	0,173

There were no significant differences in terms of concentrations of polyunsaturated fatty acids, a result considered normal, taking into consideration that diets did not have a different structure from this point of view.
Processing analytical data obtained statistically to determine the concentration of cholesterol in egg yolk (mg col / 100 g fresh yolk) were obtained differences supported statistically between the three groups (Table 6).

Table 6. Cholesterol concentration (mg col /100 g fresh yolk)

Collection	C	E1	E2
BL	0,570±0,053	0,570±0,053	0,570±0,053
Week 2	0,684±0,048	0,559±0,07	0,568±0,042
Week 8	0,559±0,13	0,519±0,053	0,520±0,034

Determinations of cholesterol content from eggs collected in the 2-nd week of the experiment differed between C group and the two experimental variants, but were not supported statistically. At the end of the experiment, data showed that in group C cholesterol concentration was significantly ($p \leq 0.05$) higher than in other groups. The results in Table 6 indicate that during experimental period cholesterol from collected eggs decreased in all groups but not as the result of given supplements administrated to the experimental groups (Poltowicz and Wezyk, 2006). This decrease was 18.27% for group C, 7.15% for group E1 and 8.45% for group E2. To explain this differentiation of yolk cholesterol concentration in the

two experimental groups compare to C group, we quote Qureshi et al. (1986). These authors demonstrated that tocotrienols (found especially in seeds) have hypocholesterolemic action. Marigold Oily Extract, made from flowers, has a certain content of tocotrienols.

CONCLUSIONS

Concerning zootechnical parameters were significant differences ($P \leq 0.05$) in terms of: average daily consumption of combined fodder, specific consumption of combined fodder/ kg egg weight, number of eggs and egg weight of group E1 compare to E2 and C. In terms of temporally feed preservation, there was a slower decay for the two experimental groups given feed that contained antioxidants (vitamin E to E1, respectively, marigold extract to E2. There were no significant differences in the concentrations of PUFA in egg yolk, taking into consideration that the diets did not have a different structure from this point of view. During the 8 experimental weeks cholesterol from eggs collected from laying hens (age 43-51 weeks) declined in all groups studied, but more pronounced in those experimentals groups.

ACKNOWLEDGEMENTS

This paper is a result of international cooperation, FP7 REGPOT-2007-3 project Feed-To-Food GA No 207043 funded by EC and the research within the project III46012 financed by the Ministry of Education and Science, Republic of Serbia.

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FEEDING VALUE ESTIMATION OF NEW INTRODUCED FORAGE PEA (*PISUM SATIVUM* L.) VARIETIES

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ABSTRACT: The general composition, plant cell walls fiber components content, in vitro digestibility and general feeding characteristics in forage quality evaluation of green mass of forage pea (*Pisum sativum* L.) spring forms in field trial – competitive variety testing, carried out at the Institute of Forage Crops – Pleven, Bulgaria (2009-2011) were studied. Four new introduced Ukrainian high productive varieties Kamerton, Glyans, Modus, Svit and Bulgarian standard variety Pleven 4 were harvested at the three vegetative stages – budding, flowering and full pod formation. Forage quality of the whole pea plants was evaluated by the parameters of general composition, plant cell walls fiber components content, digestibility, protein and energy feeding value. The standard variety Pleven 4 characterized by highest forage quality – highest protein content 18,0%, highest digestibility 74,9% and lowest plant cell walls fiber components content: NDF 36,4%, ADF 29,02%, ADL 4,57%. The new introduced Ukrainian varieties, Glyans and Kamerton show higher forage quality: higher protein content 13,3% , lower fiber content: crude fiber 23%, NDF 42-41%; ADF 30-31,5%; ADL 5,2-5,4%, respectively and digestibility 71% in comparison with varieties Svit and Modus. Energy feeding value of standard variety Pleven 4 is highest but in the group of Ukrainian varieties the highest energy value characterized Modus and Kamerton. The standard Pleven 4 distinguish highest protein feeding value: mean values for three stages – TDP 135 g kg^{-1}, PDIN 113,2 g kg^{-1}, PDIE 101,2 g kg^{-1}, and for Ukrainian varieties – variety Kamerton: TDP 89,4 g kg^{-1}, PDIN 83,4 g kg^{-1}, PDIE 89,6 g kg^{-1}, followed by variety Glyans and variety Svit.
Key words: *Pisum sativum L., varieties, protein, fiber, digestibility, energy and protein feeding value*

INTRODUCTION

The stable tendencies in planet climate require tolerate varieties to biotic and abiotic environment factors. The introduction of new varieties, the exact area distribution and effective growing management contribute to increase biological diversity, increasing yield and quality of forage pea production. The forage pea is fed as hay, silage and haylage alone or in diets. The forage pea is high nutritive source of protein and energy in animal especially ruminant nutrition (Ellwood 1998). It is fed as hay, silage, haylage alone or in mixtures with cereals in diets. The pea crop is significant in resolving the protein problem. The green mass contain 17-20 % crude protein of dry matter, minerals, sugars (Pavlov 1996). Pea grain has high protein content: 22-34% crude protein, but straw 6-9% (Kostov&Pavlov 1999). The chemical composition, protein feeding value, plant cell walls fiber components content determining energy feeding value and forage digestibility were studied in Bulgarian and some introduced forage pea varieties in breeding and management experiments (Naydenova and Todorova, 2009; Naydenova et al., 2008a, 2010b; Kirilov et al., 2010; Naydenova et al., 2010c, 2011d; Georgieva et al., 2011).
The aim of the study was evaluation the general composition, plant cell walls fiber components content, *in vitro* digestibility and estimation of potential protein and potential energy feeding value of pea forage of new introduced Ukrainian varieties in comparison with the standard Bulgarian variety Pleven 4.

MATERIAL AND METHODS

The plant material in the investigation is whole plant spring sown forms of forage pea (*Pisum sativum* L.). The field competitive variety trial in three replications of 10 m^2 plots with sown rate 120 numbers of germinating seeds was carried out at the Second experimental field of the Institute of Forage Crops, Pleven, Bulgaria, situated in the central part of the Danube hilly plain in the period 2009-2011. The soil type is leached chernozem with the followed characteristics: pH $_{(KCl)}$ – 4,78; pH $_{(H2O)}$ – 6,45; humus – 2,85% and total N content – 0,226 mg/1000 g soil, P_20_5 – 3,32 mg/100 g soil and K_20 – 30,2 mg/100 g soil. Four new Ukrainian varieties of forage pea: Kamerton, Modus, Glyans, Svit in three phenological stages in the vegetation (1. budding stage; 2. flowering stage; 3. full pod formation stage) are studied. The standard variety used is Standard Bulgarian variety Pleven 4 of forage pea, officially acknowledged by the State variety commission in Bulgaria. The forage pea is grown by approbate technology of the Institute of Forage Crops, Pleven.
The general composition of forage pea is determined as crude protein (CP) by Kjeldahl method (N x 6,25) and crude fiber (CF) by Weende system (AOAC 2001). The plant cell walls components content in energy feeding value are determined as NDF (Neutral-detergent fiber), ADF (Acid-detergent fiber) and ADL (Acid-detergent lignin) in percent of dry matter, by systematic detergent analysis of Goering and Van Soest. Enzymatic digestibility *in vitro* of organic and dry matter (IVDMD, %) is performed by two stage pepsin-cellulase method of Aufrere (Todorov et al. 2010).
The potential energy feeding value is estimated by French system UFL-UFV (INRA 1988), on the basis of equations for legumes, according experimental values of crude protein, crude fibers and digestibility of organic matter, calculated in Bulgarian (FUM-FUG), Feed units for milk, Feed units for growth by coefficients pointed from Todorov (1997). The coefficient of digestibility of organic matter dMO *in vivo* was determined by Andrieu&Demarquilly (1989), after dependence used *in vitro* digestibility of organic matter, determined experimentally. The additional comparative characteristics were estimated energy feeding value by Dutch system (VEM-VEVI). The protein feeding value (PDIN=PDIA+PDIMN and PDIE=PDIA+PDIME) was estimated by French system (INRA 1988) by the parameters: TDP/PBD-Total Digestible Protein/Protein Brute Digestible, PDIN-Protein digestible dans l'intestine in dependence of nitrogen and PDIE–Protein digestible dans l'intestine in dependence of energy. In comparative analysis at the three development stages were evaluated individual and mean values for each pea variety and the degree of variation of the parameters for forage feeding value, according their significance for the forage quality.

RESULTS AND DISCUSSION

Forage pea chemical analysis in establishing the general composition verify highest protein and lowest fiber composition content of Bulgarian standard variety Pleven 4 (Rang 1) with the follows parameters, mean for the three development stages: crude protein 18,03%, crude fiber 21,03% and *in vitro* dry matter digestibility 74,93%, i.e. that is the highest forage quality variety, grown in Bulgarian soil and climatic conditions (Table 1). In the group of the new introduced Ukrainian varieties the variety Kamerton distinguishes the highest protein content – mean value of the three development stages 13,29% of dry matter, in difference to the others three varieties contained from 10,0 to 11,7%. Crude fiber in lowest content belongs to the varieties Glyans and Kamerton – 23%, while the other two Ukrainian varieties demonstrate closed fiber content, but 2,0% of dry matter lower – almost 25,0%. The higher forage *in vitro* dry matter digestibility of Ukrainian varieties belong to variety Modus – 72,74% (Rang 2) and variety Kamerton – 71,15% (Rang 3) (Table 3). The character trait is that there is not exact corresponding in general composition – crude protein, crude fiber and digestibility in the group of new introduced Ukrainian varieties (Table 1; Table 3).
The plant cell walls fiber components (polyosides) content is presented in Table 2 and Table 3.

Table 1. Principal composition of forage pea, spring forms new varieties, competitive variety trial, 1. budding stage; 2. flowering stage; 3. full pod formation stage, % of dry matter; R-Rang

Variety	Crude protein					Crude fiber				
	1	2	3	Mean	R	1	2	3	Mean	R
Kamerton	13,92	12,42	13,52	13,29	**2**	26,44	21,58	22,08	23,37	**3**
Glyans	12,74	10,15	11,20	11,36	4	24,90	23,08	20,83	22,94	**2**
Modus	11,03	9,02	1039	10,15	5	25,48	25,35	23,63	24,82	4
Svit	14,20	10,26	10,53	11,66	**3**	26,70	24,91	23,15	24,92	5
Pleven4	20,10	16,60	17,39	18,03	**1**	20,00	21,24	21,80	21,01	**1**
Mean	**14,40**	**11,70**	**12,60**	**12,90**		**24,70**	**23,23**	**22,30**	**23,41**	
Min	11,03	9,02	10,39	10,15		20,00	21,24	21,80	21,01	
Max	20,10	16,60	17,39	18,03		26,70	25,35	23,63	24,92	
SD	*3,42*	*3,00*	2,95	*3,11*		*2,73*	*1,87*	*1,11*	*1,60*	
CV	**23,8**	**3,51**	**23,4**	**24,1**		**11,0**	**8,1**	**5,0**	**6,8**	

Table 2. Structural fiber component contents of forage pea, spring forms new varieties, competitive variety trial, 1. budding stage; 2. flowering stage; 3. full pod formation stage

Variety	NDF					ADF				
	1	2	3	Mean	R	1	2	3	Mean	R
Kamerton	40,32	39,82	43,42	41,18	**2**	34,75	27,32	3252	31,53	**3**
Glyans	40,05	41,92	43,84	41,94	**3**	34,46	26,36	29,28	30,03	**2**
Modus	44,62	45,02	43,93	44,52	5	39,27	32,09	30,17	33,85	5
Svit	40,89	44,24	43,80	42,98	4	36,63	32,54	29,42	32,86	4
Pleven4	33,95	38,93	36,26	36,38	**1**	26,78	31,89	28,39	29,02	**1**
Mean	**39,97**	**41,98**	**42,25**	**41,40**		**34,38**	**30,04**	**29,96**	**31,46**	
Min	33,95	38,93	36,26	36,38		26,78	26,36	28,39	29,02	
Max	44,62	45,02	43,93	44,52		39,27	32,54	32,52	33,85	
SD	*3,84*	*2,66*	3,35	*3,07*		*4,66*	*2,95*	*1,57*	*1,98*	
CV	**9,6**	**6,3**	**7,9**	**7,4**		**13,5**	**9,8**	**5,2**	**6,3**	

NDF –Neutral-detergent fiber; ADF – Acid-detergent fiber, % of dry matter; R-Rang

Table 3. Lignin content and *in vitro* digestibility of forage pea, spring forms new varieties, competitive variety trial, 1. budding stage; 2. flowering stage; 3. full pod formation stage

Variety	ADL					IVDMD				
	1	2	3	Mean	R	1	2	3	Mean	R
Kamerton	6,05	4,67	5,52	5,41	**3**	69,22	74,20	70,03	71,15	**3**
Glyans	5,98	5,18	4,48	5,21	**2**	70,52	62,85	71,77	68,38	4
Modus	7,10	6,16	5,09	6,12	5	65,78	84,33	68,12	72,74	**2**
Svit	6,57	6,06	5,04	5,89	4	68,32	67,28	67,83	67,81	5
Pleven4	4,24	5,32	4,14	4,57	**1**	73,98	68,97	81,84	74,93	**1**
Mean	**6,00**	**5,48**	**4,85**	**5,44**		**69,56**	**71,53**	**71,92**	**71,00**	
Min	4,24	4,67	4,14	4,57		65,78	62,85	67,83	67,81	
Max	7,10	6,16	5,52	6,12		73,98	84,33	81,84	74,93	
SD	*1,08*	*0,62*	*0,54*	*0,61*		*3,02*	*8,23*	*5,77*	*2,98*	
CV	**18,0**	**11,4**	**11,2**	**11,2**		**4,3**	**11,5**	**8,0**	**4,2**	

ADL – Acid-detergent lignin; % of dry matter; IVDMD - *in vitro* dry matter digestibility, %; R – Rang

The significant rang in forage quality evaluation is decreasing in fiber components content, determined as NDF, ADF, ADL and increasing *in vitro* digestibility of dry matter. The structural polyosides in forage plants present from 300 to 800 g kg^{-1} (30-80 %) of forage dry matter and they are the general source of nutritional energy for ruminants, but less than 50% of them are digestible and utilized (Fahey&Hussein, 1999). The Neutral detergent fiber present the total content of plant cell walls fiber components of lignin, cellulose, hemicellulose and are laboratory parameter for prediction of forage intake by ruminants. That's why they are selection criteria in evaluation of forage crops (Casler&Vogel, 1999). The Acid detergent fiber is the fraction, contained plant cell walls lignin and cellulose which determine forage digestibility. The mean ADF content is 9-10% units lower than those of NDF and in the vegetation process follows decreasing tendency. The acid detergent lignin in

forage pea, spring forms demonstrate low values (4,57 – 6,12%), which is important premise the pea stem be flat-tened. The degree of variation of plant cell walls fiber fractions, significant in forage quality evaluation in ruminant nutrition, is high (ADL: CV 11,2–18,0%; NDF: CV 6,3-9,6%; ADF: CV 5,2-13,5), because they may be utilized as selection criteria (Table 2, 3). The highest is the coefficient of variation in budding stage.

The plant cell walls fiber components fractions as a forage quality characteristics and energy source demonstrate adequate correspondence of fiber composition and digestibility of Bulgarian standard variety as well as in the group of new introduced Ukrainian varieties. Lowest fiber components content for all type of fractions – NDF, ADF, ADL and Cellulose belong to standard variety Pleven 4 (Rang 1) (Table 2, 3). In the group of Ukrainian varieties, the lowest fiber components content demonstrate varieties Glyans and Kamerton (Rang 2 and 3) as follow by mean values: NDF 42-41%; ADF 30-31,5%; ADL 5,2-5,4%; Cellulose 25-26%, respectively. The others two varieties Svit and Modus show higher fiber composition content (Rang 4 and 5 respectively), mean by 2% of forage dry matter, as follow by mean values NDF 43-44,5%; ADF 33-34%; ADL 5,9-6,2%; Cellulose 27-28%, respectively. The mentioned two varieties show higher content of undigestible fiber component lignin (Table 3).

The basic criteria, effectuated new evaluation in forage quality is *in vitro* digestibility by enzymes, characterized by rapidity, reproducibility and heredity, small sample quantity and direct correlation by *in vivo* ruminant digestibility (Fahey&Hussein 1999). The variation range of *in vitro* digestibility usually is 100 g kg $^{-1}$ dry matter. The breeding process for high protein content led to increased *in vitro* digestibility. Long-period breeding programs for increased protein content combined with decreasing the ADF (lignocellulose) content. The combined genetic changes may increase digestibility by 16 g kg^{-1} (2,1 %). It is proved that the genetic changes in *in vitro* digestibily were due to genetic changes in plant development. The digestibility of forage pea investigated is high in all development stages (69,5 – 72,0 %) and vary slowly CV 4,2%.

The forage feeding value estimation on the basis of chemical composition and enzymatic *in vitro* digestibility is suitable method for forage quality variety evaluation when it is not possible to effectuate *in vivo* digestible trials by animals (Fahey&Hussein 1999). The forage quality evaluation by composition and digestibility corresponds with the energy feeding value estimation (Table 4). Energy feeding value of standard variety Pleven 4 is highest (Rang 1). In the group of new introduced Ukrainian varieties the highest energy feeding value demonstrates variety Modus, followed by variety Kamerton.

In comparative forage quality analysis of composition and protein feeding value between varieties, the individual and referent values, standard deviations and the degree of parameter variation are presented in Table 5, 6, 7. The Bulgarian standard variety Pleven 4 distinguish by highest protein feeding value – Rang 1: mean values from the three development stages – Total digestible protein 135 g kg^{-1}, PDIN 113,2 g kg^{-1}, PDIE 101,2 g kg^{-1}, and in the group of new introduced Ukrainian varieties – variety Kamerton (Rang 2): Total digestible protein 89,4 g kg^{-1}, PDIN 83,4 g kg^{-1}, PDIE 89,6 g kg^{-1}, followed by variety Glyans and variety Svit – both evaluated by Rang 3. The variety Modus characterized lowest protein feeding value, which unsatisfactory distinguished to those of preceded two varieties.

Table 4. Energy feeding value of new introduced varieties forage pea, spring forms, competitive variety trial, mean from seven harvests

Variety	UFL-UFV		Rang	FUM-FUG		Rang	VEM-VEVI		Rang
Kamerton	0,861	0,779	**3**	0,714	0,636	**3**	960	1978	**3**
Glyans	0,830	0,745	4	0,681	0,608	4	928	1928	4
Modus	0,877	0,773	**2**	0,730	0,674	**2**	963	1980	**2**
Svit	0,819	0,732	5	0,679	0,598	5	923	1920	5
Pleven4	0,894	0,814	**1**	0,741	0,665	**1**	1005	2048	**1**
Mean	**0,857**	**0,768**		**0,709**	**0,636**		**956**	**1971**	
Min	0,819	0,732		0,679	0,598		923	1920	
Max	0,894	0,818		0,741	0,665		1005	2048	
SD	*0,032*	*0,032*		*0,028*	*0,034*		*33*	*51*	
CV	**3,7**	**4,2**		**4,0**	**5,3**		**3,4**	**2,6**	

Table 5. Protein feeding value of new introduced varieties forage pea, spring forms, competitive variety trial, 1. budding stage; 2. flowerin stage; 3. full pod formation stage, g kg^{-1} dry matter

Variety	PDIN					PDIE				
	1	2	3	Mean	R	1	2	3	Mean	R
Kamerton	87,4	78,0	84,9	83,4	**2**	89,1	90,2	89,4	89,6	**2**
Glyans	80,0	63,7	70,3	71,4	4	87,8	78,1	85,9	83,9	5
Modus	69,3	56,6	65,3	63,7	5	81,4	89,7	81,9	84,3	**3**
Svit	89,2	64,4	66,1	73,2	**3**	89,0	81,2	82,2	84,1	4
Pleven4	126,2	104,2	109,2	113,2	**1**	104,2	94,8	104,9	101,3	**1**
Mean	**90,4**	**73,4**	**79,2**	**81,0**		**90,3**	**86,8**	**88,8**	**88,6**	
Min	69,3	56,6	65,3	63,7		81,4	78,1	81,9	83,9	
Max	126,2	104,2	109,2	113,2		104,2	94,8	104,9	101,3	
SD	21,5	18,9	18,6	19,3		8,4	6,9	9,5	7,5	
CV	**23,8**	**25,7**	**23,4**	**23,8**		**9,3**	**8,0**	**10,7**	**8,4**	

Table 6. Mean values and rang of parameters of principal composition, structural fiber component contents and digestibility of new introduced varieties forage pea, spring forms

Variety	CP	R	CF	R	NDF	R	ADF	R	ADL	R	*in vitro* DMD	R	Aritmetic rang sum	R
Kamerton	13,29	**2**	23,37	**3**	41,18	**2**	31,53	**3**	5,41	**3**	71,15	**3**	16	**2**
Glyans	11,36	4	22,94	**2**	41,94	**3**	30,03	**2**	5,21	**2**	63,38	4	17	**3**
Modus	10,15	5	24,82	4	44,52	5	33,85	5	6,12	5	72,74	**2**	26	5
Svit	11,66	**3**	24,92	5	42,98	4	32,86	4	5,89	4	67,81	5	25	4
Pleven4	18,03	**1**	21,01	**1**	36,38	**1**	29,02	**1**	4,57	**1**	74,93	**1**	6	**1**
Mean	**12,90**		**23,41**		**41,40**		**31,46**		**5,44**		**71,00**			
Min	10,15		21,01		36,38		29,02		4,57		67,81			
Max	18,03		24,92		44,52		33,85		6,12		74,93			
SD	*3,11*		*1,60*		*3,07*		*1,98*		*0,61*		2,98			
CV	**24,1**		**6,8**		**7,4**		**6,3**		**11,2**		**4,2**			

When evaluate the mean values and rang of parameters of composition and digestibility by arithmetic sum of the parameter's rang, for each one of the laboratory determined parameters, the established analysis confirm forage quality evaluation of new introduced Ukrainian varieties and their comparison to the standard variety Pleven 4 (Table 7).

Table 7. Rang of the mean values of the parameters the composition, digestibility and feeding value of new introduced varieties of spring forage pea

Variety	CP	CF	NDF	ADF	ADL	*in vitro* DMD	TDP / PBD	PDIN	PDIE	UFL	FUM	VEM	Aritmetic rang sum	R
Kamerton	**2**	**3**	**2**	**3**	**3**	**2**	**2**	**2**	**2**	**3**	**3**	**3**	30	**2**
Modus	4	**2**	**3**	**2**	**2**	**3**	4	4	5	4	4	4	41	**3**
Glyans	5	4	5	5	5	5	5	5	**3**	**2**	**2**	**2**	48	4
Svit	**3**	5	4	4	4	4	**3**	**3**	4	5	5	5	49	5
Pleven4	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	12	**1**

CONCLUSSIONS

- The Bulgarian standard variety Pleven 4 (Rang 1) characterized by highest forage quality – highest protein content 18,03%, highest digestibility of dry matter 74,93% and lowest plant cell walls fiber components content: crude fiber 21,01%, NDF 36,4%, ADF 29,02%, ADL 4,57%.
- In the group of new introduced Ukrainian varieties, Glyans and Kamerton (Rang 2 and 3) show higher forage quality: higher protein content 13,3% , lower fiber content: crude fiber 23%, NDF 42-41%; ADF 30-31,5%; ADL 5,2-5,4%; Cellulose 25-26%, respectively and digestibility 71% in comparison to varieties Svit and Modus.

- The forage quality evaluation by composition and digestibility corresponds with the energy feeding value estimation Energy feeding value of standard variety Pleven 4 is highest (Rang 1). In the group of new introduced Ukrainian varieties the highest energy feeding value characterized variety Modus, followed by variety Kamerton.
- The Bulgarian standard variety Pleven 4 distinguish by highest protein feeding value – Rang 1: mean values from the three development stages – Total digestible protein 135 g kg^{-1}, PDIN 113,2 g kg^{-1}, PDIE 101,2 g kg^{-1}, and in the group of new introduced Ukrainian varieties – variety Kamerton (Rang 2): Total digestible protein 89,4 g kg^{-1}, PDIN 83,4 g kg^{-1}, PDIE 89,6 g kg^{-1}, followed by variety Glyans and variety Svit – both evaluated by Rang 3. The variety Modus characterized lowest protein feeding value, which unsatisfactory distinguish to those of preceded two varieties.

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EFFECT OF DIFFERENT SELECTION CRITERIA ON GROWTH TRAITS AND CARCASS QUALITY IN PIGS

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ABSTRACT: Research has included four breeds (Landrace and Yorkshire as fertile) and (Pietrain and Duroc as a terminal) pigs. Selection criteria are significantly different due to the negative genetic correlation between milk yield and meatiness and positive heads between capacity and production capabilities. The test involved 558 animals of both sexes who are descended from the 43 father and 282 mothers. Carcass quality tests were performed on 144 animals, also of both sexes. The average weight of animals at the end of the fattening period was 103 kg and a standard deviation of 2.6 kg. Bones density did not differ significantly between Landrace, Yorkshire and Duroc. Pietrain bones were significantly easier. This demonstrates the viability of lower growth in this race compared to the other and longer feeding for 24 to 32 days. When it comes to the only growth was significantly lower in Pietrain animals were compared to their peers of other races. The content of meat in the carcass was approximately 57.6% in fertile breeds and Duroc 58.9%. It was not significantly different. Pietrain had 62.1% of meat in the carcass which is significantly higher than in other races. Intramuscular fat content was: 0.8% of Pietrain, Duroc 2.6% 2.0% Landrace and Yorkshire 1.8%. The level of protein as the most important parameter of meat quality was the lowest in Pietrain (20.1%), Landrace and Yorkshire were 21.8% and 22.5% and 22.9% Duroc. Selection criteria relate to the last eight generations of selection and the effects show the expected trends.
Key words: *pigs, growth traits, carcass and meat quality*

INTRODUCTION

In pig production, the most important traits from economical point of view can be as follow: *number of weaned piglets per sow per year, feed conversion, growth and meat content in carcass then protein level in meat*. To provide optimal selection effect for each of them it is important to determine genetic correlation between them and size of heritability as well. According to knowledge of negative genetic correlations between fertility or milk yield and meat content in carcass it is necessarily to developed different selection criteria or better says specialized breeds.

Since of purpose of selection effects on farm production in analysis we separate due to selection criteria two groups of breed, e.g.: fertility and milking breeds – Landrace and Yorkshire and terminal breeds, e.g.: Duroc and Pietrain. Following literature sources we can accept some trend which can depends from selection criteria, farm, year and season effect of management on the farm too.

The research was defined to analyzed selection effects during more years of selection on farms. Selection criteria were different for terminal breeds (Duroc and Pietrain) compare Landrace and Yorkshire where selection has been concentrate on litter size and milk yeald.

MATERIAL AND METHODS

The experiment has been done at 4 farms since 2000 up to 2011. We included 4 breeds, L, Y as mother line and D and P as terminal one. The following pictures give different selection criteria that were used. Average alive weight at slaughter of all animals was 103 kg and standard deviation of 2,6 kg.

Table 1. Breed structure and number of animals in trial

Breed	Sire	Dam	Progeny	No. carcasses
Landrace	11	80	196	36
Yorkshire	12	74	182	36
Duroc	10	66	102	36
Pietrain	10	62	80	36

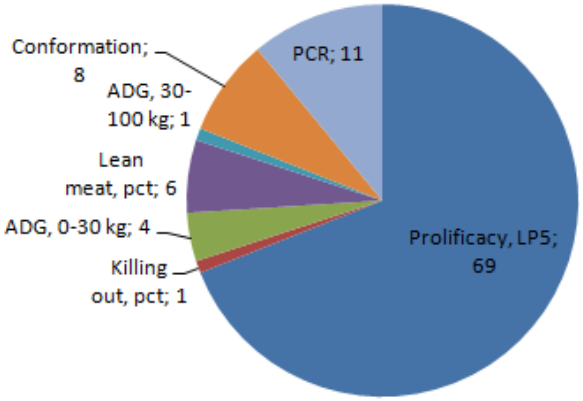

Figure 1. Used selection criteria for mother breeds: - Landrace and Yorkshire

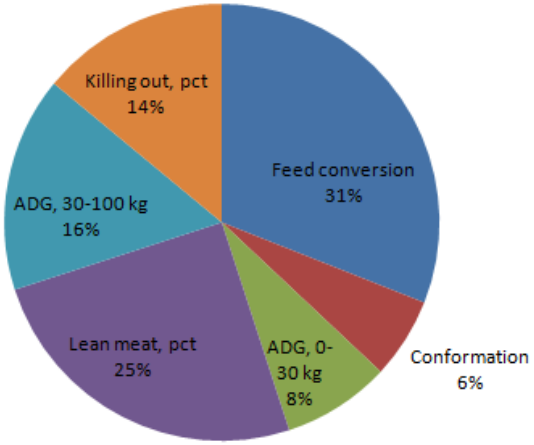

Figure 2. Selection criteria used for terminal breeds: – Duroc and Pietrain

Following MME LS model has been used to analyze influences of FYS (Farm, Year and Season) then Breed as fixed effect and Sire as random one (Vidović 2011a).

$$Y_{ijkl} = \mu + HYS_i + B_{ij} + S_{ijk} + E_{ijkl}$$

Y_{ijkl} - Number of observations hierarchically distributed;
μ - General mean of observations;
HYS_i - Fixed effect of farm, year and season;
B_{ij} - Fixed effect of different breeds;
S_{ijk} - Random sire effect;
E_{ijkl} - Residual.

RESULT AND DISCUSSION

Fattening days and feed conversion. The line (Figure 3) showed expected tendency. It means the selection criteria for most economically important traits have been well defined. Selection intensity was controlled by number of doses per jump. In case of feed conversion (FC) improvement were 90 kg per head. Most fast improvement was at first 5 years. Since that FC is average heritage there are new possibilities to continue with selection effect, e.g. to decrease FC, reduce cost and increase profit per kg of gain. Similar trend has been showed by Brascamp 1985., Rotschild 1990., Park at al. 1986., Bergsma at al. 2010., Vidović et. al. 2011a; 2011b.

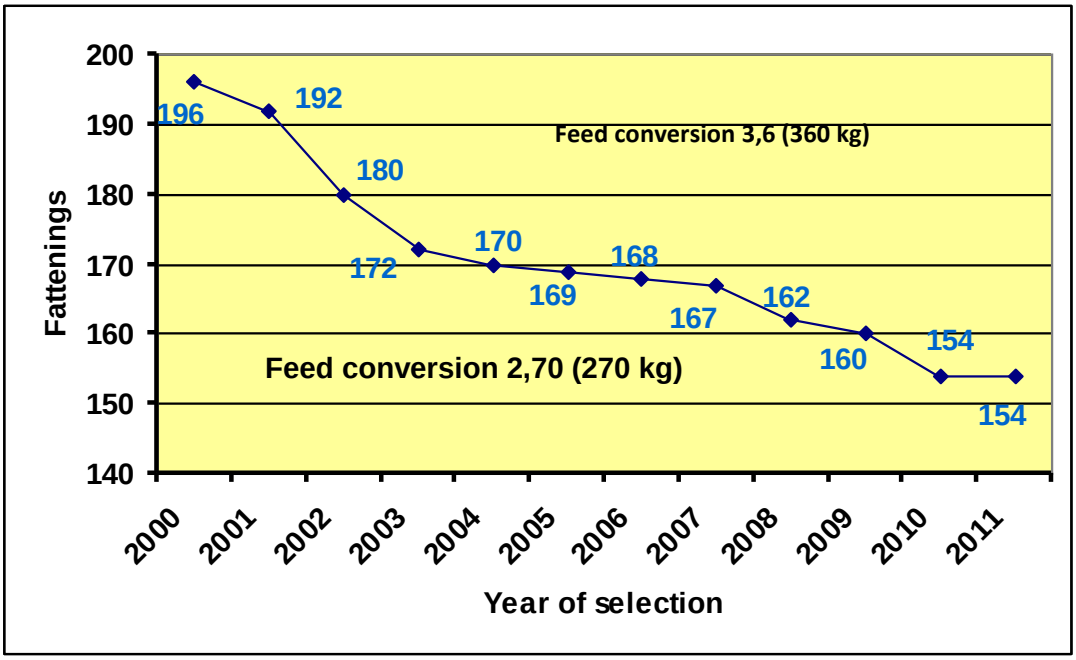

Figure 3. Effect of selection on age at slaughter (indirectly on growth) and feed conversion for Landrace, Yorkshire and Duroc

Effects:

1. Less of feed : 90 kg
2. Less fattening days : 42

Notes: There are no selection differences between Landrace, Yorkshire and Duroc. Pietrain had significantly less daily gain and age at slaughter compare to three other breeds.
According to fattening period we can recognize improvement of 42 days. Economicaly it is iprovement of about 20 euro per pig.
Comparison of age and FC between Duroc and Pietrain differences is present. Duroc had 32 days shorter period to the certain commercial weight and used 88 kg less food. Feed cost in Duroc are 19 euro less.(Figure 4). These rezult are similar to Vidović and Šubara 2010., Vidović et al. 2011c., Višnjić et. al. 2012.

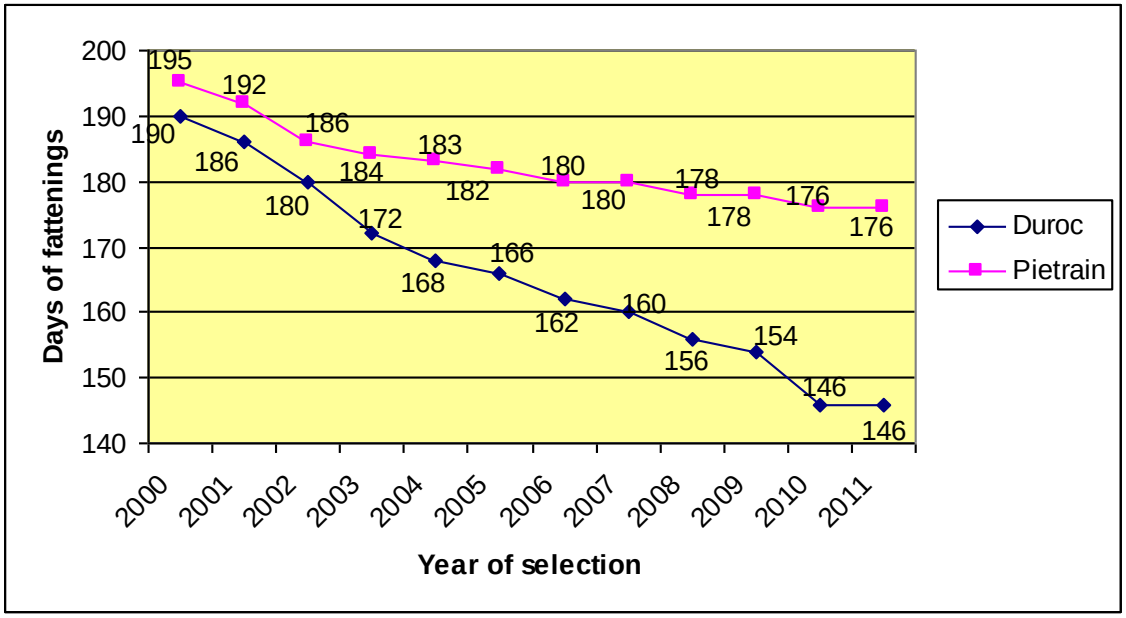

Figure 4. The differences in feed use and age at slaughter between Duroc and Pietrain in test production

The differences: Age: 32 days
Feed: 88 kg

Carcass quality. Meat content in Landrace and Yorkshire has no statistical differences even they showed optimal trend. Since those to breed are treated as dam line to provide heterosis effect at F_1 daughters selection criteria were concentrate more on fertility traits. Expected trend were very close to realized one. It is going to back fat between 16- 20 mm at that age. Later on these animals if they are going to be parents mast has some reserve to produce progeny (Figure 5).

Bones density did not differ significantly between Landrace, Yorkshire and Duroc. Pietrain bones were significantly easier. This demonstrates the viability of lower growth in this race compared to the other and longer feeding for 24 to 32 days. When it comes to the only growth was significantly lower in Pietrain animals were compared to their peers of other races. The content of meat in the carcass was approximately 57.6% in fertile breeds and Duroc 58.9%. It was not significantly different. Pietrain had 62.1% of meat in the carcass which is significantly higher than in other races. Intramuscular fat content was: 0.8% of Pietrain, Duroc 2.6% 2.0% Landrace and Yorkshire 1.8%. The level of protein as the most important parameter of meat quality was the lowest in Pietrain (20.1%), Landrace and Yorkshire were 21.8% and 22.5% and 22.9% Duroc. Selection criteria relate to the last eight generations of selection and the effects show the expected trends.

Just to remind case of present negative genetic correlations between milk yield and meat content in carcass mean that breeders have to optimize selection criteria and use specialized sire and dam lines in breeding program. In our experiment we divided into two different groups: L and Y as mother line and used totally different selection criteria compare to terminal sire lines: D and P. After 11 years of selection or 8 generations result were present (Figure 5 and 6).

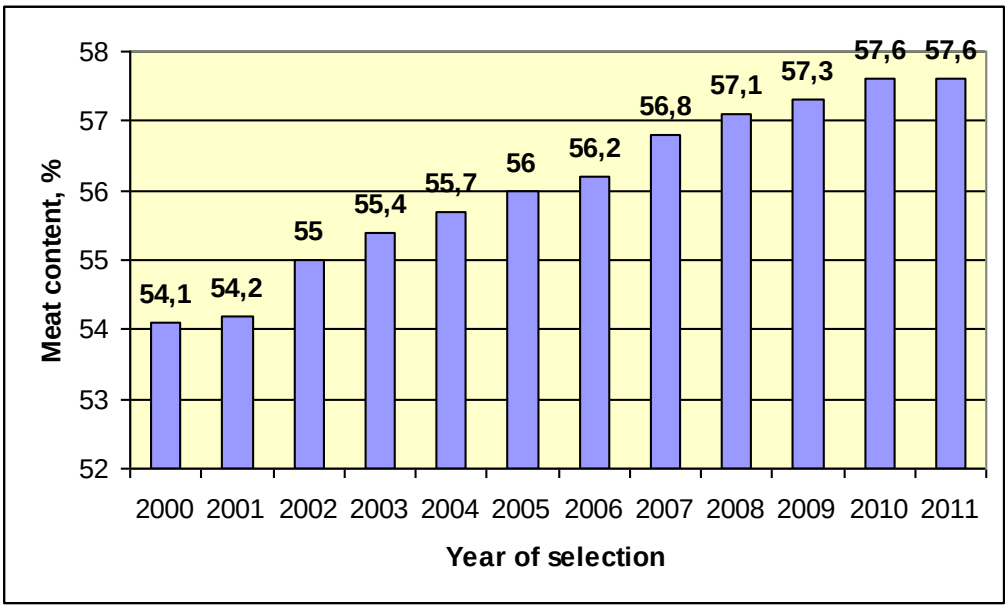

Figure 5. The trend of selection for percentage of meat content for Landrace and Yorkshire

At graph. 6 can be recognized the differences between Duroc and Pietrain even they have the same selection criteria. The only differences were at the beginning of start trial. In conclusion we can say the trend were more or les the same as selection effect. The differences of 3,4% of meat content or raptly 2,5 kg meat between them provide about 8 euro more profit in fewer to P. But in total D made about 14 euro more profit including FC and fattening period as well. Similar conclusion were defined by Gama at al 1990., Rotschild 2010., Bergsma at al. 2010., Vidović et. al. 2011c,d,e.,Višnjić et. al. 2012.

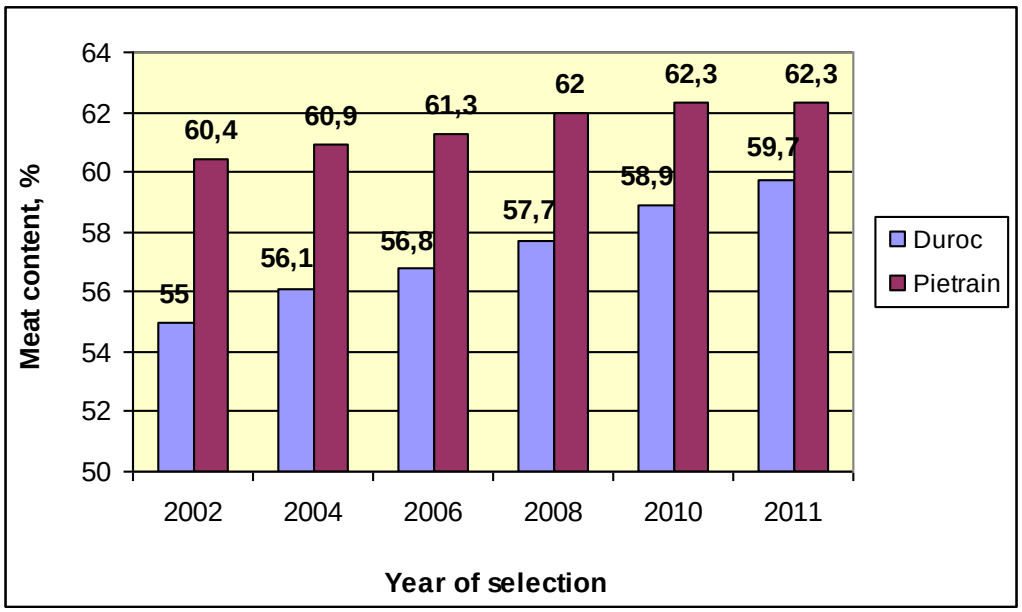

Figure 6. The effects of selection for meat content of Duroc and Pietrain

The effects in meat content, %: - Duroc: 3,9
- Pietrain: 1,9

CONCLUSION

Selection criteria for certain traits have been optimal. It showed clear genetic trend for certain traits.
There have no been selection differences for gain and age at slaughtered Y, L and D. Pietrain showed significantly les gain and needed longer period to reach certain weight. So that means more cost and less profit in case of P.
Also selection on meat content had positive trend. Selection efficiency were higher at D compare with P. Pietrain it self still have 3,4% more meat in carcass but much longer period of fattening. Intramuscular fat was much lover at P (0,5%) compare to D (2,5%). This has negative effect on meat quality in case of P.

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MILK QUALITY AND PROCESSING IN RELATION TO POLYMORPHISM OF CAPA CASEIN AT BLACK AND WHITE HOLSTEIN CATTLE

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ABSTRACT: Capa casein genotyping was performed on 36 cows, bull dams, and based on the analysis of DNA from blood using PCR – REFL techniques. Served for the identification of allelesand genotypes capa casein. Using the identified allels (A and B) and genotypes (AA, AB and BB) capa casein evaluated their frequency in the examined population of cows. Frequency capa casein genotype were: 0,450 AA +0,494 AB + 0,056 BB = 1,00. Furthermore, the frequency of allels A and B which is derived from frequencies of genotypes was 0,66 for allele A and 0,34 for allele B. This reletionship expresses the preliminary information about presence of different genotypes capa casein in Holstein. The contribution capa casein and total casein genotype on milk coagulation rate, determined by LS method, model 1, the coagulation of milk was highly significant. Using model 2, the coagulation of milk, the amount of calcium present in milk had a high impact significantly and lactation order significant impact on the speed of milk coagulation. Physical and chemical composition of fresh milk casein genotypes of different hats, meets the current criteria for the quality of fresh milk. Acceptable rennet – coagulation properties of k-BB groups are reflected in a shortetime of coagulation of milk with rennet. In particular it is a significant relatioship that manifests increased content of casein and total protein in milk. The analysis showed that reflect the time making chesse milk k-BB group was almost three times faster than k-AA milk about 2 times faster than k-AB milk.
Key words: *genetic, polymorphsiam, capa-casein, quality of milk*

INTRODUCTION

Studies of polymorphic protein systems are increasingly directed toward establishing a possible connection between the genes controlling protein polymorphisms - with other genes, desirable or undesirable, which control polygenic traits related to the productive traits of domestic animals. Determination this connection would have, undoubtedly, a great economic importance in the selection and increase productivity in livestock. Genetic variants of individual caseins differ by their structure and physico-chemical properties, of which, to a large extent on the technological properties of milk. The quality of milk has a significant effect on yield and quality of cheese, or the yield of cheese produced depends on the composition of milk. Milk must be normal to renneting curds and gives cund good features. This is possible if there are sufficient of casein and calcium, because they affect the speed of coagulation of milk under the action of rennet and the curds cund. The aim this paper was to examine the quality of milk for processing against frequency k-casein genotypes of black-white Holstein Friesian cattle. Milk proteins are expressed polymorphic nature, the application of molecular genetics techniques to identify genetic variants of these proteins. Thus, the DNA samples obtained from the blood, regardless of sex and age of the animal can be done to identify which greatly contributes to faster and more efficient selection cattle. The speed of coagulation of milk is influenced by k-casein genotype and the contents of total casein in milk, lactation number, calving season, stage of lactation, and the amount of calcium present in milk. Identification of k-casein genotype of Holstein bulls (males) has performed Pinder et al. (1991). Ng-Kwai-Hang et al. (1991) have proved that genetic forms of k-casein have to do with the speed of coagulation of milk rennet. Results Antunac et al. (1991) speaks about the impact of protein polymorphisms on milk coagulation properties, synthesis cheese cund and yield cheese. According to in-Schaar (1984) in terms of

coagulation properties, milk of BB genotype k-casein is attributed to the favourable influence. Genetic improvement of quantitative traits is relatively slow, since some productive traits can be determined only at one sex (Vidović i Lukač, 2010). Also, some can be determined only in adult animals, this is achieved by increasing the generation interval and the decrease of genetic progress by year (Vidović, 2009). For this reason, knowledge of qualitative traits, these as polymorphism of blood groups, enzymes, proteins, blood serum or different types of milk protein milk (Vidović i Stupar, 2010) provides the ability to increase the precision in assessing the genetic value of bulls and cows and selection procedures

MATERIAL AND METHODS

Kappa - casein genotyping was performed on 36 cows, of bull dams, based on analysis of DNA from blood using PCR - REPL techniques. Served for the identification of alleles and genotypes of kappa casein. In order to determine individual factors on the rate of coagulation of milk the analysis of physical - chemical composition according to the methods Carić et al., 2002. and examined the rate of coagulation under the action of rennet. The influence different factors on the rate of coagulation testing using the least squares LSMLMW, Harvey 1990.

1. Model - includes the effect of k-casein genotype, total casein, lactation number, calving season, stage of lactation and the effect of calcium present in milk coagulation rate.

$$\mathbf{Y_{ijklmn} = \mu + K - K_i + L_j + S_k + SL_l + Ca_m + e_{ijklmn}}$$

$\mathbf{Y_{ijklmno}}$ - phenotypic value of the observed trait (speed coagulation)
μ - general mean value
K – K_i – influence k-casein
L_j – influence lactation order
S_k – influence season calving
SL_l – influence stage lactation
Ca_m – influence calcium
e_{ijklmn} – random error

2. Model – include the effect of all these factors in Model 1 but without the influence of casein

$$\mathbf{Y_{ijklmno} = \mu + K - K_i + L_j + S_k + SL_l + Ca_m + b\ (X - \bar{X}) + e_{ijklmn}}$$

$\mathbf{Y_{ijklmno}}$ - phenotypic value of the observed trait (speed coagulation)
μ - general mean value
K – K_i – influence k-casein
L_j – influence lactation order
S_k – influence season calving
SL_l – influence stage lactation
Ca_m – influence calcium
b $(X - \bar{X})$ – linear regression The influence of casein
$e_{ijklmno}$ – random error

RESULTS AND DISCUSSION

DNA isolation was performed according to standard protocol using proteinase K in the presence of detergents. Clean the DNA in all samples satisfactory because the ratio of absorption at 260 nm and 280 nm ranged between 1.8 and 2.0. Table 1 shows the distribution of k-casein genotypes were of the analyzed for 36 bull dams.

Table 1. k-kasein typing DNA of bull dams

Number	Genotype	Number	Genotype	GENOTYPING	
1	AA	19	AA		
2	AA	20	AA		
3	AA	21	AA		
4	AB	22	AB		
5	AB	23	AA		
6	AB	24	AB		
7	AA	25	AB		
8	AB	26	AB		
9	AB	27	BB	AA–16	16
10		28		AB–18	18
11		29		BB–2	2
12		30		Total	36
13		31			
14		32			
15		33			
16		34			
17		35			
18		36			

During the analysis of the 16 samples genotyped with the k-casein AA genotype, 18 samples with genotype AB, and two samples as a k-casein BB genotype. The frequencies of genotypes were 0.450 +0.494 AA AB BB + 0.056 = 1.00. Frequencies of alleles A and B, which is derived from the frequency of genotypes was 0.66 for allele A and 0.34 for allele B. This ratio expresses the preliminary information about the presence of different genotypes of kappa casein in black- white Holstein cows in our population.

Table 2. Phisico-chemical composition of milk of different k-casein genotype

Genotype	pH	°SH	*Proteins* (%)	*Casein* (%)	Ca (%)	*Coagulation time* (min)
AA	6,73	6,5	2,83	2,76	0,18	8
AB	6,76	6,6	3,32	3,24	0,18	5
BB	6,76	6,5	3,93	3,84	0,19	2
Aggregate of milk	6,75	6,6	3,24	3,16	0,18	5

Based on the results shown in Table 2 can be seen that the milk cows k-casein BB genotype is characterized by a higher content of total protein or casein. Bovenhuis et al. (1992) point out that the cows k-casein BB genotype produced milk containing 0.08% higher protein content of cows genotype AA. In terms of coagulation properties, milk obtained from cows k-casein BB genotype is attributable to the favorable impact is reflected in the time of renneting milk with rennet(Table 2). Schaar et al. (1984) also concluded that the time renneting milk casein group B was 2-3 times shorter than the time renneting kasein A milk.

Table 3. F-value in determining the impact of certain factors on speed of coagulation

Trait	Model	F-value					
		k-casein genotype	Total casein	Lactation on order	Season calving	Stage lactation	Quatity of Ca
Speed of coagulation	1	34,264**	11,234**	1,134	1,117	1,277	1,135
	2	86,453	-	3,677*	1,856	2,044	5,712**

P<0,01**; P<0,05* ; P>0,05^{nz}

As seen from Table 3, using model 1, k-casein genotype and total casein have the greatest influence, and highly significant impact on the speed of coagulation of milk, while the effect of other factors, no significant influence. On the basis of model 2, which excludes the influence

of total casein in milk coagulation rate, and includes the effects of other factors studied, it can be concluded that the amount of calcium in the milk had a highly significant impact on the speed of coagulation of milk, lactation in order a significant, while other factors are not had a significant influence on the observed trait.

CONCLUSIONS

Estimated frequency of alleles A and B can be given preliminary information on the presence of different genotypes of k-casein in the studied population. Genetic variant of k-casein BB in terms of coagulation characteristics preferable to k-casein AA because it is associated with increased content of casein, k-casein and total protein in milk. Favorable rennet coagulation properties of milk k-BB groups are reflected in a shorter time of coagulation of milk rennet. Time renneting milk k-BB group was about 2 times faster than other k-casein group. So, preferably increase the frequency of type B k-casein, as it directly favors the selection. In particular, rapid progress can be made in the selection of bulls maintain homozygous k-casein B gene.

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THE INFLUENCE OF BREED AND SEX ON TISSUE DISTRIBUTION IN PIG CARCASSES

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ABSTRACT: The aim of this study was to determine differences in the distribution of certain tissues (muscle and adipose tissue and bone) in carcasses of male and female animals. The paper included two fertile breeds: Yorkshire and Landrace and Hampshire breeds as terminal. Carcass dissection was performed by model EU 1992. During dissection data was collected about: weight of animals at slaughter, weight hot and cold carcass, less valuable parts, weight and proportion of the back, the neck, thigh, belly, shoulders the carcass, and the share certain tissues (muscle, fat and bone) on the carcass. Carcass weight in male animals was 40.66 kg with fat thickness on the back of 24.54 mm and 30.58 mm on the ramp, while in the female animal carcass weight was 40.37 kg, the thickness of fat on the back 21.49 mm and 26.71 mm on the ramp. Comparing the proportion of muscle tissue in the carcass of male and female animals was statistically significant difference ($P < 0.05$). Comparing within the breed, no statistically significant differences ($P > 0.05$) in the percentage of meat between two fertile breeds (Landrace and Yorkshire), while there is a statistically significant difference ($P < 0.05$) between the two fertile breeds and terminal (Hampshire). The share of meat in the carcass of female animals was 56.94%, while the males had a lower percentage of meat, 54.82. Sex and breeds have highly significant effect on the amount of meat on the carcass.
Key words*: pigs, sex, quality carcasses*

INTRODUCTION

One the most important trait from economical point in pig production is meat content in carcass. A good quality carcass regardless of breed or sex characterized high proportion of muscle tissue in the carcass. Differences between the sexes in pigs are of special importance, because sex influences the composition of pig carcasses, and the quality properties of muscle tissue (Evans and Kempster, 1979., Petričević et al. 2000., Kušec et al., 2002.). Many studies indicate that there may be some differences between the populations of pigs (resulting from the use of different selection criteria and in accordance with the objectives of the market) in relation to carcass composition and tissue distribution (Evans and Kempster, 1979., Vidović,1987.,Planella and Cook, 1991., Gu et al., 1992., Engel and Walstra, 1993., Daumas and Dhorne, 1997.) According to market demands, pork carcass must be accompanied by a higher proportion of muscle tissue of satisfactory quality, and a smaller proportion of fat. Evaluation meatiness and classing of carcasses of slaughtered pigs is based on the determination of carcass composition (ratio of tissue in them) and some that are significant for commercial value. Given the importance of meatiness in pig carcasses, the aim of this study was to determine differences in the distribution of certain tissues (muscle and adipose tissue and bone) in carcasses of male and female animals.

MATERIALS AND METHODS

This paper included two fertile breeds (Landrace and Yorkshire) and a terminal breed (Hampshire). Carcass dissection was performed on the model of EU 1992. The study involved 327 randomly chosen pig carcasses classified by breed and sex: 191 (93 males and

98 females) breed Landrace, 59 (30 males and 29 females), breed Yorkshire and 77 carcasses (27 male and 50 female) breed Hampshire. During dissection data was collected about: weight of animals at slaughter, weight hot and cold carcass, less valuable parts, weight and proportion of the back, the neck, thigh, belly, shoulders the carcass, and the share certain tissues (muscle, fat and bone) on the carcass. The measured length of the carcass, the average value of backfat thickness measured at the middle of the back and of the ramp. Average slaughter weights of female animals were 102.20 kg and 103.22 kg male.
Statistical analysis was performed using the software Statistic 10. To determine the difference between the average values of individual groups the Duncan test was used. To determine the influence of sex and breed to the observed properties the ANOVA was used.

RESULTS AND DISCUSSION

Values of the quality of pig carcasses are shown in table 1. We can see that the average warm carcass weight, carcass length and backfat thickness measured at the back and ramp. With the average carcass weight in male animals from 40.66 kg in back fat thickness was 24.54 mm and 30.58 mm ramp, while in the female animal carcass weight was 40.37 kg, with the thickness of fat on the back 21, 49 mm and ramp 26.71. Males animals had greater fat thickness (3.87 mm) on the back and ramp in relation to female, both within breed and between breed. Female animals were on average had longer carcasses (0.63 cm) in all three breeds compared to males. Also, backfat thickness was greater in males of 3.87 mm compared to the females.
Comparing the proportion of muscle tissue in the carcass of male and female animals was statistically significant difference (P <0.05) (table 2).Comparing within the breed, no statistically significant differences (P> 0.05) in the percentage of meat between two fertile breeds (Landrace and Yorkshire), while there is a statistically significant difference (P <0.05) between the two races and terminal fertile Hampshire (table 3). To the same results came Kušec et al. (2002, Petricevic et al. (2000.) who were found a statistically significant difference between the breeds and between sexes within a breed.

Table 1. Parameters of the quality value of pork carcass sides

Parameters	Indicator	Landrace		Yorkshire		Hampshire		Sex	
		♂	♀	♂	♀	♂	♀	♂	♀
Weight of warm carcass sides, kg	$\overline{X}$	80,73	80,04	81,26	80,62	83,44	82,20	81,32	80,74
	S	4,91	4,67	5,41	2,93	6,87	5,67	5,47	4,82
	V	6,09	5,83	6,66	3,36	8,23	6,90	6,72	5,96
Lenght of carcass os pubis – atlas, cm	$\overline{X}$	96,56	97,32	96,11	96,22	96,84	97,37	96,52	97,15
	S	2,50	3,19	1,76	2,66	2,62	2,57	2,39	2,96
	V	2,59	3,28	1,84	2,76	2,71	2,64	2,48	3,04
Back fat thickness - back fat,mm	$\overline{X}$	25,19	21,45	23,70	21,17	23,25	21,76	24,54	21,49
	S	4,23	4,09	3,77	3,60	4,23	3,75	4,20	3,90
	V	16,81	19,06	15,94	17,03	18,22	12,25	17,13	18,17
- ramp fat,mm	$\overline{X}$	31,40	26,96	30,07	26,07	28,34	26,24	30,58	26,71
	S	3,71	4,24	4,29	3,47	4,26	3,73	4,08	3,98
	V	11,84	15,75	14,26	13,03	15,04	14,24	13,39	14,90

Table 2. Mean values and standard error of mean values by sex for the amount of meat in the carcasses

Sex	n	$\overline{X}$	SD $\overline{x}$
♂	150	21,76 ^{a}	0,156
♀	177	22,57 ^{a}	0,313

P < 0,01 – same smol letters

Table 3. Mean values and standard error of mean values by breed for the amount of meat in the carcasses

Breed	n	$\overline{X}$	SD $\bar{x}$
Landrace	191	21,82 ^{a}	0,137
Yorkshire	59	22,27 ^{a}	0,213
Hampshire	77	23,07 ^{b}	0,194

P < 0,01 – different smol letters; P > 0,05 – same smol letters

Proportion of muscle and backfor tissue, bone and less valuable parts are shown in table 4. The results showed that the proportion of meat in the carcasses of females animals 56.94%, 26.73% fat, bone, 9.53% and less valuable parts of 6.80%. Unlike female animals, males had a lower percentage of meat in the carcass (54.82%), higher percentage of fat tissue (29.15%), percentage of bone was 9.25% and less valuable parts of 6.78%. The share of fat and bones were no significant differences between the sexes. To the same results came and Petricevic et al. (2000), who were also found no statistically significant difference between male and female animals in the proportion fat and bone.

Table 4. Proportion of muscule, backfar and bones in carcass (kg)

Share	Indicator	Landrace		Yorksire		Hampshire		Sex	
		♂	♀	♂	♀	♂	♀	♂	♀
Muscle tissue	$\overline{X}$	21,38	22,24	21,83	22,73	22,96	23,13	21,75	22,57
	%	54,25	56,69	54,73	57,22	56,78	57,13	54,82	56,92
	S	1,86	1,83	1,79	1,33	1,73	1,71	1,91	1,76
	V	8,71	8,25	8,22	5,86	7,55	7,39	8,78	7,81
Backfor tissue	$\overline{X}$	11,77	10,57	11,59	10,49	10,83	10,73	11,56	10,60
	%	29,86	26,94	29,06	26,40	26,78	26,50	29,14	26,73
	S	1,37	1,64	1,61	1,19	1,54	1,65	1,48	1,57
	V	11,64	15,56	13,88	1,42	13,99	15,40	12,83	14,87
Bones	$\overline{X}$	3,58	3,73	3,76	3,77	3,86	3,87	3,67	3,78
	%	9,08	8,59	9,42	9,49	9,54	9,56	9,25	9,53
	S	0,26	0,35	0,39	0,31	0,29	0,31	0,32	0,34
	V	7,51	9,59	10,52	8,28	7,55	8,07	8,79	9,04
Less valuable parts (head,legs,tail,kidney)	$\overline{X}$	2,67	2,68	2,70	2,71	2,76	2,74	2,69	2,70
	%	6,77	6,83	6,77	6,82	6,82	6,76	6,78	6,80
	S	0,19	0,29	0,19	0,18	0,21	0,23	0,19	0,26
	V	7,22	10,95	7,18	6,76	7,71	8,73	7,35	9,75

The influence of sex and breed on the amount of meat in pig carcasses are shown in table 5. We see that all the factors (sex and breed) have a highly significant impact on the amount of meat on the carcass. To the same results came Kušec et al. (2008.), Latorre et al. (2004.), Ball et al.(1996), Evans and Kempster (1979.).

Table 5. Influence of sex and breed on the amount of meat on the carcass

Source of variation	DF	SS	MS	F	P
Sex	1	73,5	36,8	11,66	0,0000**
Breed	2	40,6	12,89	12,89	0,0003**

P < 0,01** – highly significant; DF – degree of freedom; SS – sum of squares; MS – middle of the squares;
F – value; P - probability

The share of the more important parts of pig carcasses originating from male and female animals are shown in table 6. It can be seen that between the sexes within breed and between the breeds there were no significant differences in cold carcass weight and percentage of representation of certain parts of the carcasses. The same conclusions are there Kušec et al. (2008.), Ball et al.(1996). These results coincide with the results of Latorre et al. (2004.), who did not identify statistically significant differences in cold carcasses weight in male and female animals. The largest and most important share in the carcasses, but it ham with a share of 29.10% in males and 29.51% in female animals. To similar data arrived and Kušec et al. (2008.), Vidović et al., (1994. 2011.) where the share of ham in carcass was been 25.15% for males and 25.00% in female animals.

Table 6. Proportion of major parte of the pork carcass side

Carcass side part	Indicator	Landrace		Yorksire		Hampshire		Sex	
		♂	♀	♂	♀	♂	♀	♂	♀
Cold carcass weight	$\overline{X}$	39,37	39,18	39,85	39,69	40,37	40,44	39,74	39,89
	S	4,68	5,39	5,30	2,71	6,59	5,36	5,15	5,22
	V	5,89	6,88	6,61	3,41	8,04	6,61	6,49	6,52
Lenght	$\overline{X}$	6,50	6,55	6,41	6,54	6,70	6,67	6,53	6,58
	%	16,51	16,17	16,08	16,47	16,59	16,49	16,37	16,55
	S	0,56	0,59	0,57	0,49	0,67	0,61	0,59	0,58
	V	8,68	9,05	9,00	7,54	10,11	9,14	9,08	8,85
Neck	$\overline{X}$	3,08	3,18	3,18	3,30	3,27	3,38	3,17	3,28
	%	7,82	8,11	7,97	8,31	8,10	8,35	7,94	8,25
	S	0,30	0,36	0,28	0,42	0,47	0,35	0,34	0,37
	V	9,87	11,33	8,82	12,78	14,45	10,33	10,89	11,57
Ham	$\overline{X}$	11,62	11,72	11,57	11,64	11,65	11,85	11,61	11,73
	%	29,51	29,91	29,03	29,32	28,85	29,30	29,10	29,51
	S	0,85	0,87	0,85	0,48	0,74	0,75	0,83	0,79
	V	7,32	7,47	7,41	4,20	6,41	6,40	7,14	6,74
Ribs of the abdominal	$\overline{X}$	6,83	6,55	6,89	6,71	6,91	6,85	6,87	6,70
	%	17,34	16,71	17,28	16,90	17,11	16,93	17,22	16,85
	S	0,51	0,60	0,62	0,45	0,59	0,85	0,54	0,67
	V	7,59	9,18	9,07	6,70	8,29	12,48	7,99	10,09
Shoulder	$\overline{X}$	6,62	6,71	7,15	6,96	7,07	7,07	7,01	6,91
	%	16,81	17,12	17,94	17,53	17,48	17,48	17,57	17,38
	S	0,50	0,78	0,67	0,37	0,57	0,57	0,67	0,69
	V	7,66	11,73	9,40	5,39	8,17	8,17	9,78	10,14
Less valuable parts (head,legs,tail, kidney, lard, throat)	$\overline{X}$	4,72	4,47	4,65	4,54	4,56	4,62	4,64	4,54
	%	11,98	11,40	11,66	11,43	11,29	11,42	11,63	11,42
	S	0,16	0,17	0,16	0,14	0,18	0,17	0,16	0,17
	V	15,01	16,68	15,66	14,86	17,24	17,35	16,58	17,09

CONCLUSIONS

From the results, can be seen that the average carcass weight in male animals was 40.66 kg with fat thickness on the back of 24.54 mm and 30.58 mm on the ramp, while in the female animals carcass weight was 40.37 kg, the thickness of fat on the back 21.49 mm and 26.71 mm on the ramp. Fat thickness was greater in males of 3.87 mm. Comparing the proportion of muscle tissue in the carcass of male and female animals was statistically significant difference ($P<0.05$). Comparing within the breed, no statistically significant differences ($P>0.05$) in the percentage of meat between two fertile breeds (Landrace and Yorkshire), while there is a statistically significant difference ($P<0.05$) between the two fertile breeds and

terminal (Hampshire). This is so because the selection criteria are different. The share of meat in the carcass of females animals was 56.94%, while the males had a lower percentage of meat, 54.82%. At the share fat and bones were no significant differences between the the sexes. Between the sexes within breeds and the between breeds there was no significant difference in cold carcass weight and percentage of representation of certain parts. Sex and breeds have highly significant effect on the amount of meat on the carcass. The capacity of animals is of particular importance how at fertile so that terminal breeds. Quality rating of pigs and forming of prices at slaughter line is a prerequisite for the establishment of trust between farmers and butcher. The motive of these relationships is the profit because farmers by performing a selection on meatiness (it is known that when the pig growth in the kg meat must consumed about 1.7 kg of concentrate while in the example growth in kg of fat consumed about 7.0 kg) to earn more money to save in the food while butcher buy animals with more optimum meat quality.

ACKNOWLEDGEMENTS

Research was part of the project TR - 031032 financed by the Ministry of Science and Technological Development, Republic of Serbia.

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POLYMORPHISM AND EFFECT OF CAPA CASEIN ON TECHNOLOGICAL QUALITY MILK FOR TRAPIST CHEESE PRODUCTION

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ABSTRACT: The main objectives of study were to evaluate the impact of milk polymorphism significantly protein, k-casein, and to investigate whether it is possible to use this influence to improve milk production. The purpose were to examine how technology and in terms of milk processing can take advantage of these impact. The k-casein genotyping was performed in 18 cows. Isolation of DNA was performed in all blood samples. Determination of k-casein polymorphism in Red and White Holstin cattle based on analysis DNA using PCR-RFLP methods. We have analyzed the chemical composition and physical properties of milk obtained from cows of different k-casein genotypes (AA, AB, BB) and the aggregate of milk for production of cheese. Genotyping of cows has been identified 8 animals with AA, 9 with AB and 1 BB k-casein genotype. The frequencies of genotypes were: 0,44 AA + 0,500 AB + 0,056 BB = 1. Frequency of allels A and B were estimated from frequencies of genotypes, so the frequency of allel A was 0,69 and the frequency of allel B 0,31. It was found that the physicochemical composition of fresh milk that was used to produce trappist according to the criteria for the quality of milk. There were significant differences in milk fat content that are over the values for milk fat used to produce cheese. In neither sample of milk or aggregate milk not found the remains of mycotoxins and antibiotics, or *E. Coli*. The total number of microorganisms and somatic cells satisfying our standards.
Key words: *polymorphism k-casein, technological quality of milk, Trappist cheese*

INTRODUCTION

Over the last several decades have gained important knowledge about the polymorphic properties of a large number of proteins of body fluids. Thanks primarily electrophoretic studies, it was found that the most important constituents of casein: α-casein, β-casein and k-casein, exhibit characteristics of polymorphic proteins. The study of genetic polymorphism of milk proteins has, in addition to the general biological, genetic and biochemical importance, also, the importance of technology for animal and milk production. Polymorphism of k-casein studied Woychik (1964), Leeson et al. (1988), Bech and Kristiansen Rotvig (1990), Pinder et al. (1991). The main objective of this study was to determine the most advantageous genetic variants of k-casein milk from which it is possible to improve the quality and milk production traits for the production of Trappist cheese. Possessing pleiotropic effects of milk protein genes (Vidović i Lukač, 2010; Vidović i Stupar, 2010) affect the technological properties, primarily on the properties related to the production of cheese. The relationship between polymorphism of milk proteins and cheese yield were studied Marziale and Ng-Kwai-Hang (1987). Making of Trappist cheese is not well balanced yet and there is great variability in organoleptic and chemical properties (Herczegh and Fenyves, 2001). However, no matter, we can say that here is a Trappist cheese, which are typical and optimal organoleptic and chemical properties of partially and clearly defined by the quality of milk Rules.

MATERIAL AND METHODS

Kappa casein genotyping was performed in 18 cows (bull dams). DNA isolation is the first step in the analysis and manipulation of DNA. The insulation is made from all 18 blood samples, with 14 samples isolated using standard procedures (Sambrook et al., 1989), and 4 samples with off-the DNA kit. Determination of k-casein polymorphism in red and white Friesian cattle based on analysis of DNA (from blood) using PCR-RFLP method. Amplified DNA products were analyzed by electrophoresis. On the basis of identified alleles and genotypes was a calculated frequency of alleles and genotypes in examined population. In order to study the effect of different k-casein genotypes on the technological quality of milk for the production of Trappist cheese, fresh milk (different k-casein genotypes and aggregate milk) used for this purpose should meet the current criteria for the quality of fresh milk, according to the quality of milk Rules. When analyzing the chemical composition and physical properties of milk obtained from different k-casein genotypes (AA, AB, BB) and cumulative milk following parameters were determined: dry matter, milk fat, ash, total protein, titration acidity (original or natural acidity), casein, content non casein nitrogen, lactose, calcium, volumetric weight, cholesterol content, phosphorus, sodium, somatic cells, the total number of microorganisms, the identification of *E. coli*, antibiotics and mycotoxins.

RESULTS AND DISCUSSION

Isolation of DNA was performed according to standard protocol using proteinase K in the presence of detergents. Clean DNA in all samples is satisfactory because the ratio of absorption at 260 nm and 280 nm was between 1.8 and 2.0. Using amplification of DNA fragments of 453 bp by PCR-RFLP method was performed to identify alleles A and B, k-casein. Table 1 shows the distribution of k-casein genotypes for 18 bull dams analyzed by PCR-RFLP method. During the screening analysis genotyping the 8 samples with k-casein AA genotype, 9 samples with AB and 1 sample as k-casein genotype BB. The frequencies of genotypes were 0.444 AA + 0.500 AB + 0.056 BB = 1 The frequencies of alleles A and B were estimated from genotype frequencies, so that the frequency of allele A was 0.69 and the frequency of allele B 0.31. Considering the relatively small number of samples, however, these data can provide preliminary information on the presence of different genotypes of k-casein in red and white Holstein cows.

Table 1. K-casein DNA typing of bull dams

Ser. Numb.	Genotype	Ser. Numb.	Genotype	GENOTYPING	
1	AA	10	AA		
2	AA	11	AA		
3	AA	12	AA		
4	AB	13	AB	AA–8	8
5	AB	14	AA	AB–9	9
6	AB	15	AB	BB–1	1
7	AA	16	AB	Total	18
8	AB	17	AB		
9	AB	18	BB		

Test results of physicochemical composition of milk from cows of different k-casein genotypes used for the production of Trappist cheese are shown in Tables 2 and 3.

Table 2. Chemical composition and physical properties of milk for the production of Trappist cheese

The parameters	Aggregate of milk	Milk AA k-casein genotype	Milk AB k-casein genotype	Milk BB k-casein genotype
Dry matter (%)	13,10	13,11	13,32	11,90
Milk fat (%)	3,95	4,28	4,32	3,61
Specific gravity (˚LD)	1,030	1,032	1,032	1,031
Acidity (˚SH)	6,70	6,50	6,20	6,60
pH	6,73	6,80	6,83	6,78
Proteins (%)	3,13	2,91	3,01	3,17
Lactose (%)	4,85	4,50	4,67	4,43
Casein (%)	2,58	2,55	2,57	2,64
Dry matter fat free (%)	9,15	8,83	9,00	8,29
Ash (%)	0,79	0,71	0,79	0,75
Ca (%)	0,19	0,17	0,19	0,18
Na (%)	0,058	0,06	0,07	0,06
P (%)	0,11	0,10	0,095	0,11
Fe (mg/kg)	0,41	0,45	0,40	0,43

Table 3. Parameters of importance to the technological quality of milk for the production of Trappist cheese

The parameters	Aggregate of milk	Milk AA k-casein genotype	Milk AB k-casein genotype	Milk BB k-casein genotype
Tests of antibiotic	Without antibiotic	Without antibiotic	Without antibiotic	Without antibiotic
Cheese fermentation tests	Compact curd	Compact curd	Compact curd	Compact curd
Cholesterol (mg/g)	2,52	3,07	3,12	1,71
Mycotoxins	Without mycotoxins	Without mycotoxins	Without mycotoxins	Without mycotoxins
Total bacterial	0,5 x 10	1,0 x 10	1,0 x 10	0,8 x 10
Escherichia coli	Not found	Not found	Not found	Not found
Somatic cells	164.000	340.000	227.500	155.000

From the results of determining the basic indicators of physical and chemical composition of crude milk used to produce Trappist, it is evident that the milk fat content in milk AA and AB genotype k-casein is above the value used for the production of Trappist cheese (According to the Regulations up to 4% milk fat) and due to the use of milk obtained from the evening milking when milk fat content is higher than the content of the morning milk. The dry matter content of fat-free AA and AB k-casein genotype was within permitted limits, while the value of k-casein BB genotype were below the prescribed value. (Regulation 2002 at least 8.50). Milk casein genotype BB cap is characterized by a higher protein content compared to the AA and AB genotype. Beskorovajni et al. (2002) found that milk BB-type k-casein containing 0,13% milk protein than AA type. Lactose content was within the limits of known values. Minerals are very important for the technological properties of milk and its behaviour during the treatment and processing. In this regard the most important are calcium and phosphorus. One of the calcium is bound to the casein (calcium caseinate), the second part is related to the form of phosphoric acid and phosphate insoluble absorbed from casein, while only 31% of the total amount of calcium are insoluble salts. This percentage increases with increasing acidity of milk. An average sample of milk of all genotypes and aggregate milk contained the value of minerals and acidity within the allowed limits. The examination of certain parameters that are relevant to making quality of milk obtained from cows of different k-casein genotypes (AA, AB and BB) and aggregate milk that is used in the production of Trappist cheese was found that in any sample of milk have not found the remains of mycotoxins and antibiotics, or E. Coli. This suggests that the milk produced in hygienic conditions and that it is a milk of healthy cows. Microbiological status of milk is very important for the technological quality of

milk for cheese (Popović et al., 2009). The total number of bacteria in all samples meets the standards of regulation. Bacteriological status of milk indicates that milk used to produce Trappist cheese made with respect to hygiene during milking, transport and treatment of milk. It also shows that the milk is properly cooled after milking; the milk is cooled to low temperatures slow down or stops the activity of microorganisms. Milk casein genotypes (AA, AB and BB) and aggregate milk with rennet form a compact and sturdy curd. The number of somatic cells in 1 ml of milk in accordance with the Rules, which suggests that milk is a healthy cow. Based on these results, can see that the cholesterol content was significantly increased in samples of milk AA and AB k-casein genotypes, which is consistent with the content of milk fat in the same samples. Been recorded from the given tables that the milk BB k-casein genotype used to produce Trappist better technological quality in terms of protein and casein in milk compared to the AA and AB k-casein genotype.

CONCLUSIONS

Genotyping of the cow k-casein has been identified as two k-casein alleles: A and B, using PCR-RFLP method. K-casein DNA typing 18 bull dams identified 8 individuals with AA, 9 AB and 1 with BB k-casein genotype. The frequency of alleles A and B were estimated from the frequencies of genotypes, so the frequency of allele A was 0.69 and the frequency of allele B 0.31. These results may provide preliminary information on the presence of different genotypes of k-casein in red and white Holstein cattle. Physical and chemical composition of fresh milk that is used to produce Trappist cheese meets the criteria for the quality of fresh milk. However, there were significant differences in milk fat content as they were above values used for the production of Trappist cheese. Not one sample of milk or aggregate milk not found the remains of mycotoxins and antibiotics, or *E. Coli*. This shows that the milk produced in hygienic conditions and that it is a milk of healthy cows. The total number of microorganisms and somatic cells satisfies our standard. Milk k- milk casein BB genotype were used to produce Trappist is better in terms of technological quality protein and casein in milk compared to the AA and AB k-casein genotype.

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BACTERIA IN ANIMAL FEED AND THEIR SUSCEPTIBILITY TO ANTIMICROBIAL DRUGS

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ABSTRACT: Control of microbiological safety of animal feed is regulated by a law. The regulation is based on methodological standards for estimation of feed quality. Those methods are permanently amended and with a strive for continuous improvement of microbiological safety of feed. The question remains whether the presence or absence of some bacterial species is sufficient for proper assessment of feed safety. The answer to this question can be found not only in the examination of the number of bacteria in animal feed, but also in qualitative analyzes of the isolated microorganisms. The sensitivity of bacteria from animal feed to antimicrobials is one of the qualitative characteristics of microorganisms that can affect food safety even if the feed meets the standards and is within the maximum permitted level. The subject of our work was to determinate the presence of bacteria in animal feed with a purpose to examine if they possess lower sensibility to antibiotics. For testing antibiotic sensitivity the isolates from routine microbiological feed control were used. The animal feed was analyzed by the standard methods for the isolation and identification of bacteria and determination of susceptibility was done using disc diffusion method according to CLSI. The following bacteria were detected: *Escherichia coli*, *Enterobacter* sp., *Staphylococcus* sp., *Micrococcus* sp., *Bacillus cereus*, *Bacillus* sp. The results showed that some isolates have reduced susceptibility to certain antimicrobial drugs. This finding gives a reason for further examination of certain characteristics of the bacteria found in animal feed and detect their possible influence on ecology.
Key words: *animal feed, bacteria, antimicrobial drugs*

INTRODUCTION

Different types of microorganisms can be found in feed. The microorganisms can be transmitted in different ways from the ground: by wind, rain and insects or by mechanical treatment (K.G. Maciorowski et al., 2007). Since the feed is the first critical point in the food production chain, it is necessary permanently to control the feed in terms of its safety. A detailed microbiological control of feed is described in the current legislation (“Official Gazette” SFRJ 25/1980). This implies also to the methods of testing the number of microorganisms. In this way an entry of certain pathogens and zoonotic microorganisms in the feed is prevented. In an indirect way, this ensures the microbiological safety of food of animal origin which can become a potential source of human infection. The standard methods for feed control and allowed limits in terms of number of saprophytic microorganisms are available.

The number of saprophytic microorganisms permitted by regulation is different depending on the type of feed and the animal category for which the feed is intended. Legal acts determine only the quantity of saprophytic bacteria but the qualitative characteristics of saprophytes can be overlooked. On the other hand, saprophytes are living organisms that can posses some undesirable properties. They can also transfer some of their properties to microorganisms in the digestive tract of animals. A qualitative analysis of saprophytic bacteria can contribute in preventing the transfer of undesirable features. An example is the transfer of resistance genes to microbes that reside in animals. The increased selection of the resistant bacteria in animals and humans and the spread of genes that carry these characteristics may affect ecosystem (Leila Soufi et al., 2011). For these reasons, the subject of our research was

related to monitoring the presence of bacteria in animal feed in order to determine whether the isolated bacteria posses the decreased sensitivity to antibiotics.

MATERIAL AND METHODS

Animal feed was analyzed for six months in the second half of 2011. The examined samples originated from feed mills that regularly check their products. A total of 26 samples was examined for different animal categories. Total mix used in poultry fattening and growing breeding hens (starter, grower, finisher), as well as the mix for calves and food supplements (vitamin and mineral, vitamin and protein) were examined. The samples were processed according to the methodology described in the Ordinance on the methods of performing microbiological analysis and super nutritional support life ("Official Gazette" SFRJ 25/1980). The samples were homogenized and weighed to 20 g in the Erlenmeyer flask bottle. A A sterile physiological saline solution was added to the volume of 180 mL to obtain a basic dilution 10^{-1}. From this dilution 1 ml was taken and placed directly on blood agar (agar with 5% defibrinated sheep blood) and McConkey agar. Identification of bacterial isolates was done after Gram stain and microscopic examination was performed by determining the physiological characteristics of bacteria through a series of biochemical tests (Quinn et al., 2002).
The sensitivity of isolated strains was done as recommended by CLSI (2006). We used antibiotic disks Tetracycline (30 μg), ampicilline (10 μg), streptomycin (10 μg), Ceftriaxone (30 μg), Trimethoprim + sulfamethoxazole (25 μg (trimetoprim 1,25 μg + sulamethox-azole 23.75 mg)), chloramphenicol (30 μg), lincomycin (2 μg), enrofloxacin (5μg), amoxicillin (30 μg (amoxiciline 20 μg + 10 μg clavulonic acid)) (produced by Bioanalyse) while Lincospectin (109 μg (lincomycin 9 μg + Spektinomicin100 μg) discs were from the producer Oxoid.

RESULTS AND DISCUSSION

From a total of 26 processed materials 62 isolates were detected. Within the total number of isolated bacteria six different bacterial species were confirmed. Our study is limited to the obtained isolates and the sensitivity to antimicrobial drugs is given in Table 1.
Among *Enterobacteriacae* most frequently *Escherichia coli* and *Enterobacter sp* were found. These microorganisms were represented with 62.85% of total isolates. *Bacillus sp., Staphylococcus sp.* and *Micrococcus sp.* are microorganisms that are widespread in nature and are part of the saprophytic microbiota of humans, animals and the external environment. *Flavobacter sp*. belongs to a group of microorganisms that are found as commensals in the soil and their findings may indicate that the feed is contaminated and has been, directly or indirectly, in contact with bacteria of the soil.
The results showed that *E. coli* and *Enterobacter sp*. as the predominant microorganisms, contaminating feed, carry a high resistance to certain antimicrobial drugs. These two species showed complete resistance to lincomycin. This can be due to the long use of antimicrobial products as growth promoters or in prophylactic purposes (Frank M. Aarestrup, Henrik C. Wegener 1999., Torrence E. Mary 2001). An extraordinary high resistance (75-100%) to lincomycin and the fact that 50% or more isolates were resistant to ampicillin, amoxicillin and lincospectin is surprising since it is obvious that the strains isolated from food animals were in contact with the antimicrobial drugs long enough to develop resistance. Equally important would be to determine the sources of microorganisms from animal feed because of their characteristic high resistance to certain antimicrobial drugs that can occur only in contact with these agents or through horizontal transfer of the genome responsible for resistance (K.G. Maciorowski et al., 2007, Velhner Maja et al. 2010). The data obtained are similar to the study from Portugal (Paulo Martins da Costa et al., 2007) where the resistance in *E. coli* was relatively low (29.9% ampicillin, tetracycline 27.7%) or absent for amoxiclav. The study also shows that the resistance to three and more antibiotics was present in 18.1% of isolates.

Table 1. Sensitivity of isolated bacteria to antimicrobial therapy

	E. coli			*Enterobacter sp.*			*Bacillus sp.*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
1. Tetracycline	33,3	27,7	38,8	27,5	29,2	43,3	75	8,33	16,67
2. Ampicillin	33,3	11,1	55,5	26,4	19,1	54,5	41,66	16,67	41,66
3. Streptomycin	50	38,8	11,1	48,2	41,4	10,4	66,66	8,33	25
4. Ceftriaxone	100	-	-	91,3	8,7	-	50	8,33	41,66
5. Trimetho.+sulf.	100	-	-	93,5	6,5	-	33,33	16,66	50
6. Chloramphenic.	94,4	-	5,55	89,1	4,2	6,7	58,33	16,6	25
7. Lyncomicin	-	-	100	-	-	100	25	-	75
8. Enrofloxascine	100	-	-	100	-	-	91,66	-	8.33
9. Amoxyclav.	38,88	5,55	55,55	36,15	6,05	57,8	66,66	-	33,33
10. Lyncospectino.	-	44,44	55,55	-	39,15	60,85	25	41,66	33,33

S – Sensitive, I – Intermediate, R - Resistant

Table 1. Sensitivity of isolated bacteria to antimicrobial therapy (continued)

	Flavobacter sp.			*Staphylococcus sp.*			*Micrococcus sp.*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
1. Tetracycline	100	-	-	83,3	16,6	-	91,6	8,4	-
2. Ampicillin	80	-	20	83,3	16,6	-	92,7	7,3	-
3. Streptomycin	80	-	20	83,3	16,6	-	82,9	17,1	-
4. Ceftriaxone	80	-	20	66,6	16,6	16,6	89,3	10,7	-
5. Trimetho.+sulf.	80	20	-	83,6	-	16,6	82,5	8,5	9
6. Chloramphenic.	100	-	-	83,3	16,6	-	83,3	16,6	-
7. Lyncomicin	20	40	40	42,8	28,63	28,57	50	38,3	11,7
8. Enrofloxascine	80	10	-	85,7	14,3	-	87,1	12,9	-
9. Amoxyclav.	80	20	-	85,7	14,3	-	75,8	24,2	-
10. Lyncospectino.	20	60	20	71,42	-	28,5	68,2	15,4	16,4

S – Sensitive, I – Intermediate, R - Resistant

Microflora of the digestive tract contains hundreds of bacterial species (S. Steve Yan, Jeffrey M. Gilbert, 2004). The vertical transfer of resistance determinants can occur via plasmids, prophages, transposons and integrons (S. Steve Yan, Jeffrey M. Gilbert 2004, Velhner M. et al., 2010), especially among Gram negative bacteria. Subsequently drug-resistant strains can be transmitted from animals to humans. The presence of bacteria from the family *Enterobacteriacae*, such as *E. coli* and *Enterobacter sp*. in animal feed may contribute to the spread of resistance genes through horizontal or vertical transfer. This is why bacteria carrying resistance genes, become part of the microflora of the digestive tract (S. Steve Yan, Jeffrey M. Gilbert 2004). Also, resistance to some antimicrobial drugs can occur in herds that were not in contact with drugs during their lifetime.
The presence of resistant strains of *Flavobacter sp*. in animal feeds confirms the possibility of transfer of soil bacteria to feed (K.G. Maciorowski et al., 2007). Also the observed resistance to some antimicrobials may indicate that the antibiotic preparations came to the soil where they caused the resistance. On the other hand it is possible that the emergence of resistance occurred as a result of the spread of bacteria among which the horizontal resistance gene transfer may happen.

CONCLUSION

Control of feed in respect to quantitative microbiological examination meets the standards that provide the limits of permissible and impermissible microorganisms. Our study has shown that the qualitative analysis of bacterial isolates contributed to a higher degree of food safety since it can provide information about the reduced sensitivity of bacteria to antimicrobial agents, a possible elimination from the food chain which leads to improvement of ecology.

ACKNOWLEDGEMENTS

Research was financed by the Ministry of Science and Technological Development, Republic of Serbia, project TR 31071.

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THEORY AND PRACTICE OF EXPLOITATION OF FEED MILLS OF THE IV GENERATION

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ABSTRACT: The article presents the analysis of work of feed mills, which use a portioned grinding. It is shown that the components such as extraction cakes of olive cultures and limestone contain a significant amount of mealy fractions, which don't require grinding. The scheme of technological process suggests the preliminary sifting of the portions of components that are directed on grinding with the aim of separation the mealy fraction. The use of an additional mixer is justified to obtain a preliminary mixture of microcomponents that improves the uniformity of their distribution in the mixed feeds.
Key words: *technology of production of the mixed feed, feedmills, chart of technological process, efficiency*

INTRODUCTION

The end of last century was marked by the beginning of building of feed mills of IV generation for which characteristic feature is portioned grinding of components (Kersten et al., 2003; Iegorov, 2011). In the European Union, the USA, Indonesia, Brazil, Argentina and China dozen of such feed mills were constructed for last 20 years. Today in Ukraine 7 feed mills of IV generation are exploited. Such schemes are typical for variety of such leading machine-building companies as Buhler AG (Switzerland), Andritz Feeds and Biofuel (Denmark), VanAarsen (Netherlands), Awila anlagenbau GmbH (Germany), Wynveen International (Netherlands) and allows to make loose and the pelleted mixed feed. According to the scheme a portion of the weighed components (grain, extraction cakes of olive cultures, limestone) arrives into portioned grinding knot which represents the overgrinding bunker, hammer grinder and the filter-cyclone established on the special bunker. The grinded portion of components is submitted to the main mixer with the help of the conveyor. Portion of the weighed protein components can also just be submitted to the main mixer, and can be sent to the portioned grinding knot (in case, if the size of particles of these raw materials exceed the allowable limit for the produced mixed feed limits). As a result of such technological operations the performance of the feedmill is significantly reduced.
Microcomponents such as premixes, amino acids, enzymes, adsorbents, etc. are unpacked manually to the bunkers of the technological line of microdosing. Microcomponents are weighed in turn in accordance with the prescription of mixed feed and sent to the main mixer. As a result of mixing we get loose mixed feeds According to the information of the manufacturers of the process equipment, used mixers provide the uniform distribution for microcomponents in the composition of mixed feeds in the ratio of 1:100000 (Charlton et al., 2007). However as research has shown in practice, dosages for microcomponents may reach the minimum values (up to 0,05 %) of the mass of the recipe feed which questioned the possibility of uniform distribution of such component in the mixture (McEllhiney, 1994). In addition in most cases vegetable oil is put to the technological line of the main mixing, which complicates the process of obtaining highly uniform mixture. Receiving of results of non-uniform mixed feed can become the reason of decrease in productive action.
Technologies of the IV generation are characterized by the high flexibility of the technological process, the low specific energy consumption for production of mixed feed, the high quality and productive effect of mixed feed. Further improvement of the technology of production of

the mixed feed requires the theoretical foundation and practical confirmation of the proposed solutions. Primarily the technological process of grinding portions of the mixed feed requires the improvement as some of them contains fractions that does not require grinding. As the result, there is an overgrinding of components such as soybean extraction cakes or limestone.
The experience of the industrial operation of the constructed feed mills in combination with the analysis of the peculiarities of the raw material base and the differences in the organization of production and the results of new researches allows us to formulate a number of suggestions to improve the efficiency of production of mixed feed.

MATERIAL AND METHODS

Assessment of the efficiency of grinding of mixed feed components was carried by the method of particle size analysis of the particles.
Cost of energy for production of mixed feed was determined by relating the installed power to the performance and stability of the technological process, and it was evaluated by the index of stability, which is defined as the ratio of the minimum and maximum variance of the distribution of controlled quality index.
Content of the mealy fraction was determined in soybean extraction cakes and limestone. Energy consumption on the grinding portions of feed components and on the production of one ton of the ready-mixed feed was also determined.

RESULTS AND DISCUSSION

Primarily the technology of portion grinding requires improvement as oilcakes, extraction cakes of olive cultures and limestone consists are in the portion. They contain a considerable amount of mealy fractions (Figure 1).

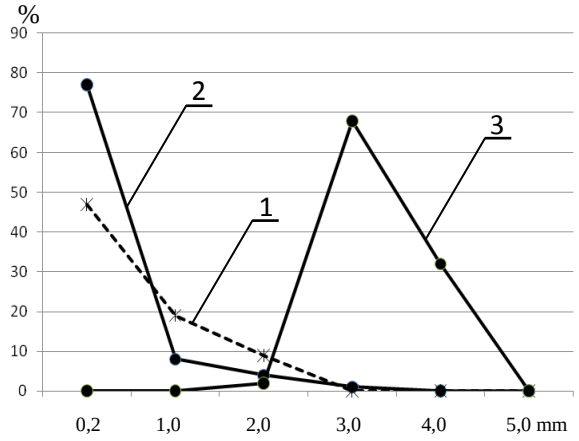

Figure 1. Granulometric composition of
limestone (1), soybean extraction cake (2) and

Their overgrinding leads to waste energy and reduce operational durability of the basic working elements of hammer grinders. The investigated limestone (Zakupansk deposit, Ukraine) contains 77,4 % of the particles of size less than 0,56 mm and soybean extraction cakes - 43,25 % of the particles of size less than 0,56 mm. It is reasonable to use preliminary sifting of the portions of the weighed components before sending them to the portioned grinding knot. Such a decision is consistent with the findings obtained in the course of the research, conducted by Nasi and Calini (1997).
The question of the uniform distribution of the portion of microcomponents in the loose mixed feed rises acutely. It is necessary to provide the installation of a paddle mixer to obtain a preliminary mixture of such components as products of synthetic amino acids, adsorbents,

acidifiers and other biologically active substances which are included in mixed feed in extremely small dosages (about 0,05 %). The necessity of this step is justified in the work of Iegorov (2011). In addition, installation of the additional mixer enables to receive highly concentrated vitamin and mineral premixes (blend) and pre-mixes for the subsequent manufacture of mixed feed.

The question of application of technological systems of microspraying liquid preparations of biologically active substances, first of all enzymes, again requires scientific-practical substantiation because their application had previously been justified by the use of heat-labile dry biologically active substances. At the present time their thermal stability has increased substantially due to the introduction of nanotechnology production of matrix microcapsuled enzyme preparations. The use of technological processes of steam-conditioning and expansion of mixed feed before granulation is not in doubt as sanitary purity of the components of mixed feed is worse in recent times.

Thus you can submit an improved scheme of technological process of production of the mixed feed of IV generation (Figure 2) by realizing the above suggestions. The proposed scheme allows making the following kinds of products: loose mixed feed, pelleted mixed feed, grits mixed feed, expanded grits mixed feed, protein-vitamin additives and pre-mixtures of microcomponents.

The maximum productivity is 20-60 t/h of pelleted mixed feeds. During the production of protein-vitamin additives and pre-mixtures productivity is reduced in relation to the chosen prescription.

Grain raw material comes by railway and motor transport. It is cleaned and placed in storage as a rule in the existing elevator. Oilcakes and extraction cakes may come loose and be placed in storage in the warehouse floor-type and in silos. Wheat bran can be received from the mill with the help of pneumatic transport.

Limestone (grits, flour) is in the big-bags and in a bulk form and it can be unloaded by use of the receiver from rail transport. Storage is provided in the floor type warehouse. Feed to the production is carried out by loader with the help of the receiving device. It is advisable to use a separate system to download the limestone in silos 59-70.

Protein components (meat, meat and bone, fish flour, fodder yeast) are also placed on the storage in the warehouse floor storage and fed to the production with the help of a separate receiver to the overweighing bunkers. It is possible to feed the limestone to some of these bunkers.

Packed raw materials (premixes, amino acids, enzymes, adsorbents, etc.) are placed on the storage also in the floor type warehouse and fed to the production with the help of the freight elevator to the overweighing bunkers of the micro-dosing line.

Liquid preparations of enzymes are fed to the production also with the help of the freight elevator on the floor where microspraying system is located.

Grain components, oilcakes, extraction cakes and limestone are weighed with the help of a multi-component weighing batcher. First the weighted portion is fed to the first operational bunker, and then it is fed to the second operational bunker. The presence of two operational bunkers allows providing stable work of management system of the portion grinding. It is recommended to sift components before grinding. This provides separation of mealy fraction which is sent to the bunker under the hammer grinder. A large fraction comes to the hammer grinding and a small fraction goes to the undergrinding bunker. It is expedient to install a rotary shutter before undergrinding bunker in order to avoid violations of the trajectory of the material flow of the products of the grinding process in undergrinding bunker and dusting.

Preliminary sifting allows substantially (for 25-50 %) improving the productivity of hammer grinder, to reduce the unit cost of electricity for grinding and to increase the operation life of the kit hammers. Grinded portion of the components is sent with the help of transporter 5 to the mixer 1. Protein raw materials located in the overdosing bunkers are dosed in accordance with the recipe and also with the help of transporter 5 are sent to the mixer 1.

If these components require grinding, they are immediately placed in these bunkers or sent to the line of portioned grinding with the help of transporter 3. The plant's capacity can be significantly reduced.

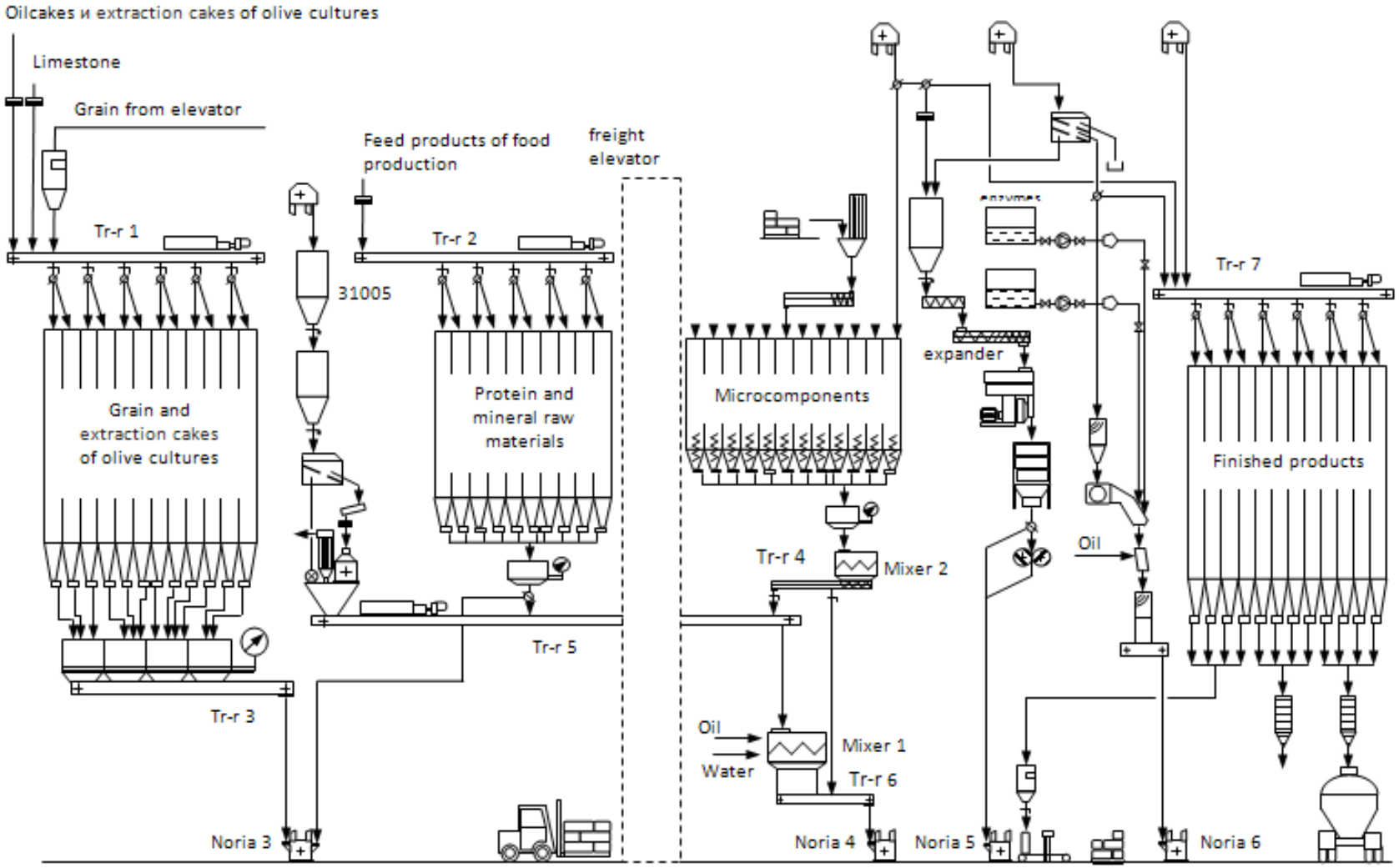

Figure 2. Improved scheme of the technological process of production of the mixed feed of the fourth generation

On the line of micro-dosing weghing for microcomponents is located in the overdosing bunkers with a capacity of 500-1000 litre each of them. Limestone or any other component of animal feed (soybean or sunflower extraction cake, fodder chalk, etc.) can be used as filler. The weighted portion of microcomponents is directed to the mixer 2 and to a mixer 1 using a transporter 4 and 5 after the reception of a homogeneous mix.
After mixing, feed is sent by transporter 6 and noria 4, in the bunkers of finished products 61-70 or on the line of pelleting. In the case of production of pre-mixes there is the possibility of feeding mixture from mixer 1 to the separate bunker for microdosing line which allows bringing highly concentrated form of feed products to the required concentration by a subsequent mixing with the filling.
Mixed feed is sent to the expander before pelleting which allows improving the hygiene quality, digestibility of nutrients, efficiency of the process of pelleting and increasing the operation life of the pellet press. It is possible to obtain whole pellets or crumbles by crumbling granules in the rolling grinder and the subsequent sifting. It is possible to apply of liquid preparations of heat-labile enzymes on the surface of granules or crumbles. The scheme provides the possibility of production of mixed feed in packaged form.
The scheme is enhanced by magnetic protection to improve the quality of finished products and to avoid sparking because mounted in the hammer grinders and pelletisers magnetic separators are not clearly sufficient in the production of mixed feed from a contaminated fodder raw materials.
During the construction of the new feed mills it is necessary to take into account a number of factors specific to each country. For example, the level of development of transport infrastructure, logistics and information support essentially influence on the determination of optimal terms of storage of raw materials and finished products, which in turn largely determine the cost of the project.
As a result of studying of possibilities of Ukrainian suppliers of fodder raw materials and efficiency of functioning of the national transport infrastructure we determine the expected timing of storage of some types of raw materials and finished products. For example, it is assumed to have a supply of grain up to 2500- 5000 tons to feed mill with capacity of 20 tons per hour. The storage time of bran wheat should not exceed 2 days. Coming from the mill bran wheat have a humidity of about 15,0-15,5 %, that causes threat of sticking of raw materials in silos. It is necessary to provide an opportunity to move the bran from one silo to another.
The expected storage periods of oilcakes and extraction cakes of olive cultures are: the oilcakes - from 5 to 14 days, the extraction cakes - from 14 to 30 days on storage in silos. The expected dates for storage of fodder products of food and biochemical industries are from 10 to 14 days.
Limestone and feed chalk can come by the motor transport (in big-bags of 1 ton or bulk) and with a railway transportation in big bags or in bulk. Salt comes in polypropylene bags with capacity of 25 to 50 kg. The expected storage periods are: limestone - from 5 to 14 days (in winter), feed chalk - from 5 to 14 days, phosphates - from 7 to 30 days, salt sodium - from 5 to 14 days.
The expected storage periods of premixes and other microcomponents are from 7 to 14 days.

Liquid types of raw materials. It is supposed to use sunflower oil and soya oil and liquid concentrate of biologically active substances with the installation of the micro-spraying line. The expected storage periods are from 7 to 30 days.

Finished products. It is supposed to manufacture loose or pelleted mixed feed (in bulk or packed form in four-layer paper craft-bags or woven polypropylene bags weighing 5, 10, 20 and 40 kg). The expected dates of storage of mixed feed in silos up to 70% of the capacity of the plant are 3 days and the expected dates of storage of packed mixed feed are up to 30 days. It is proposed to use warehouse floor storage with area 1200m^2 for their placement, storage and delivery.

A characteristic of Ukrainian feed mills is redistribution of the production capacity. About 30-50 % of the production capacity is provided for the production of mixed feed for their own needs because virtually all new factories are included in the composition of agro-holdings. The remaining production capacity is used for the production of mixed feed for sale for a small wholesale and retail for small farmers. Regarding this it is expedient to set up two pelletisers on the line of feed pelleting. For example, it is advisable to install one of the pelletisers with a capacity of 15 t/h and the second - with a capacity 5-8 t/h when the capacity of the feed mill is 20 t/h. Such step is profitable because you can acquire a larger number of dies of different sizes to press a lesser productivity that allows meeting consumer demand for a wide range of pelleted feeds, without frequent replacement of matrix at the main pelletiser.

CONCLUSIONS

Improved scheme of technological process has been implemented in the construction of a feed mill in Rozdelnaya (Odessa region, Ukraine). In the course of the conducted research, it is advisable to install sieve separators before hammer grinder for separation of mealy fraction of portions of the components that require on grinding. As a result, the energy consumption for production of mixed feed can be reduced by 10-15 % and the stability of the technological process increases from 0,85...0,90 to 0.95...0,97. Three year experience of operation of the feed mill showed high efficiency of proposed technical and technological solutions which can be used in the construction of new feed mills.

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INFLUENCE OF MYCOTOXINS IN SWINE FEED ON THE HEALTH STATUS OF PIGLETS

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ABSTRACT: Mycotoxins are secondary metabolites of fungi that can contaminate animal feeds at all stages of food production chain. Consumption of feed contaminated with mycotoxins may result in immunosuppresion, which represent a factor predisposing livestock to infectious diseases. From the epidemiological point of view, it is important to note that mycotoxins may cause breakdown of active immunity and occurence of disease even in properly vaccinated animals. The aim of the paper was to evaluate the influence of mycotoxins on the health status of piglets. The material for this research included the samples from five swine farms, where health disorders in suckling and weaned piglets were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: epidemiological and clinical evaluation, pathomorphological examination, standard laboratory testing for detection the precence of aerobic and anaerobic bacteria, virological testing and microbiological feed testing, in order to examine the presence of fungi and mycotoxins by the method of thin layer chromatography. In our research the persistant presence of various infections, which react poorly or do not react on the applied antimicrobial therapy was discovered. The presence of mycotoxin in feed can be directly connected to the detected health disturbances in piglets on the examined swine farms (vulvovaginitis, pneumonia, gastroenteritis).
Key words: *mycotoxins, swine feed, piglets diseases*

INTRODUCTION

Mycotoxins are secondary metabolites of mould, and so far, approximately 400 secondary metabolites with toxigenic potential produced by more than 100 moulds have been reported (Kabak et al., 2006). At the global level, it is considered that 25% of the world crop production is contaminated by mycotoxins, which may be a risk factor affecting human and animal health (Bouhet and Oswald, 2005). The issue of mycotoxins and mycotoxicoses in veterinary medicine is directly connected to the usage of mouldy and adversely storage of diffent types of grains (corn, wheat, barley) in animals feed (Osweiler, 2006).
In swine production in our geographic region, the most common are mycotoxicosis caused by zearalenon (F-2 toxin, ZEA), but also aflatoxins (AF), ochratoxin (OCT) and trichothecenes. (Gonzales and Rodriguez, 2008). The clinical and patomorphological picture of mycotoxicoses in swine depend on the age and category (breeding animals, suckling and weaned piglets, fatteners) (Prodanov et al., 2009). The young animals are much more sensitive to the effects of mycotoxins comparing to adults. Mycotoxins have hepatotoxic, nephrotoxic and immunosuppressive effects (Kabak et al., 2006), which can further complicate clinical and pathomorphological picture and diagnosis of mycotoxicosis in swine. The biggest challenge with mycotoxicoses is the non-specific nature of symptoms in the affected animals. Consequently, the health disorders due to mycotoxins in the feed are difficult to diagnose (Osweiler, 2006; Prodanov et al, 2009). It has been recognised by veterinary clinicians that marked immunosuppression is observed in livestock ingesting mycotoxins at levels below those that cause overt toxicity (Oswald et al., 2005). Immunosuppressive effects of mycotoxins are of special interest and may have significant influence on the occurence of infective diseases of pigs (Obremski et al., 2008; Prodanov-Radulović et al., 2011).

The aim of the paper was to evaluate the influence of mycotoxins on the health status of suckling and weaned piglets.

MATERIAL AND METHODS

The material for this research included the samples from five swine farms, where health disorders in suckling and weaned piglets were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: epidemiological and clinical evaluation, pathomorphological examination, standard laboratory testing for detection the precence of aerobic and anaerobic bacteria, virological testing and microbiological feed testing, in order to examine the presence of fungi and mycotoxins by the method of thin layer chromatography.

RESULTS AND DISCUSSION

In the first examined farm, the health disturbances in suckling piglets and weaners were registrated. Clinically, the diarrhoea in suckling piglets already in the first 3 days of life after farrowing were detected. After supervision of the farm records several facts were discovered: diarrhoea occurs in the piglets of normal birth body weight, the percent of mortality is higher in animals in good body condition and on the weaning there is 30% of small piglets. Therapeutic treatment of piglets by oral and parenteral antibiotics aplication did not improve health problems. On the farm dams are twice vaccinated during gestation with the aim to prevent disease in piglets (diarrhoea) in the first days of life. By clinical examination the certain number of suckling piglets the clinical sign of vulvovaginitis (swelling and reddening of the vulva) were dicovered. Carriyng health control in the weaned piglets the diarrhoea and signs of pneumonia (cough, nasal secretion) were detected. The pathomorphological examination of the dead suckling piglets revealed lesions dominantly on the mucosal surface of the digestive tract (*Haemorrhagiae mucosae ventriculi, Enteritis catharralis acuta*). In dead weaners beside lesions in the digestive tract, the prominent pathological changes in lungs were discovered (*Pneumonia fibrinosa in statu hepatisationis rubrae et griseae*). By bacteriological testing on tissue samples deriving from dead animals the following bacteria was detected: *Escherichia coli haemolytica, Streptoccocus alfa haemolyticus, Pasteurella sp*. Having in mind the clinical and pathological symptoms observed, especially the signs of vulvovaginitis in just farrowed piglets, a justified suspicion on the presence of mycotoxins in feed was made. Microbiological first feed for piglets testing detected 3-fold increase in the number of fungi genera *Fusarium sp, Penicillium, Aspergillus, Rhisopus sp*. as compared to the level set by the regulation. Applying further laboratory testing increase of the total number of fungi in the large number of examinated feeds was discovered: corn ($887x10^3$ *Aspergillus, Rhisopus*), piglets second feed ($319x10^3$ *Aspergillus, Mucor, Rhisopus*), feed for pregnant sows ($123x10^3$ *Penicillium, Fusarium*) and feed for lactating sows ($526x10^3$ *Aspergillus, Penicillium, Mucor*). The presence of mycotoxins was detected: zearalenon (ZEA) in the feed for pregnant sows (0.72 mg/kg), aflatoxin (AF) B1 in the piglets first feed (0,018 mg/kg) and ochratoxin (OCT) A in the piglets second feed (0.12 mg/kg).

On the second examined swine farm the health problems included increased incidence of clinical and pathomorphological signs of infective diseases. Analysing the existing data on the farm, the high mortality in piglets 7 days before weaning was noticed, which do not decrease after medical treatment. With the aim to overcome the problem, the measure of medical treatmet of piglets 3 days before weaning was introduced, but again with no result. In the weaned piglets the disease was clinically characterised with the signs of severe yellowish diarrhoea, dehidratation, huddling, roughly hair and sporadically coughing. Therapeutic treatment of the diseased animals was multiple: the antibiotics were given through feed, water and parenterally. Appying pathomorphological examination on the dead weaned piglets, the prominant changes on the digestive and respiratory tract were detected (*Gastroenterotyphlitis haemorrhagica, Poliserositis fibrinosa massiva, Pneumonia complex*).

By bacteriological testing on tissue samples from dead piglets the following bacteria were isolated: *Escherichia coli haemolytica, Pasteurella sp, Streptococcus uberis*. By laboratory feed testing i. e. the available first feed for piglets the presence of OCT (0.5 mg/kg) and ZEA (4 mg/kg) was discovered.

The presence of mycotoxin in feed for pregnant sows have influence on the occurence of decreased immunological defence in piglets (Prodanov et al., 2009). As a consequence of immunosuppresive action of mycotoxins, clinical and pathological lesions correspond to the infective diseases of different ethiology (Kabak et al., 2006; Obremski et al., 2008). Combinations of several mycotoxins may potentiate the action of one other, or at least exert an additive effect (Osweiler, 2006). Nutritional effects associated with feed refusal may also contribute to observed decreased efficacy of therapeutic treatments and vaccination (Oswald et al., 2005). In the second evaluted case, the presence of OCT and ZEA in the swine feed was detected. Consequently, on the farm an evident decrease in the swine immunity against infective diseases was noticed and there was no positive respond on the applied antibiotic therapy. Also, the occurence of diarrhoea and increased percent of waste piglets can be connected with presence the mycotoxins in the feed, because the piglets display clinically feed refusal. The gastrointestinal tract represents the first barrier against ingested food contaminants and natural toxins. Following ingestion of mycotoxin contaminated feed, intestinal epithelial cells could be exposed to a high concentration of toxin (Bouhet and Oswald, 2007). From a public haelth perspective, increased infections in animals may result in increased animal-to-human transmission of pathogens and/or increased antibiotic concentrations in meat, as a consequence of animal treatment (Oswald et al., 2005).

The complex health problems on the third swine farm were noticed. Applying control of the anamnestic data, lately the frequent periods when sows delivery mummified piglets, stillbirths and decreased litter size were observed. Beside this, just farrowed piglets are nonviable and despite the medical treatment, they live only 3-4 days after birth. In the pregnant dams the immunoprophylaxis is carring out with the aim to prevent the outbreak of disease in suckling pihglets. Beside this, the antibiotics are added in the sows feed 7 days before and 7 days after farrowing. Sporadically, the occurence of severe yellowish diarrhoea in piglets and apparent clinical signs of vulvovaginitis in just born piglets were evident. In the weaners the clinical signs of diarrhoea and pneumonia are sporadically noticed. The pathomorphological examination of the dead weaners revealed lesions on the organs of respiratory (*Pleuropneumonia actinobacillosa, Pneumonia interstitialis*) and digestive tract (*Gastroenterotyphlitis haemorrhagica acuta*). Applying bacteriological examination on the tissue samples deriving from dead piglets the following bacteria were isolated: *Escherichia coli haemolytica, Pasteurella sp, Actinobacillus suis*. After laboratory testing of swine feed samples the simultaneous presence of several mycotoxins was established: ZEA (6.4 mg/kg), AF (0.0064 mg/kg), OCT-A (0.032 mg/kg).

For the known mycotoxins of clinical importance for swine production, the response is usually subacute or chronic and the presenting signs are often subtle and vague (Gonzales and Rodriguez, 2008). The continuos intake of small amounts of mycotoxins leads to chronic intoxication which is clinically characterized by the loss of weight, insufficient weight gain and increased susceptibility for infectious diseases (Osweiler, 2006). Ingestion of low doses of mycotoxins can increase intestinal colonization by opportunistic pathogenic bacteria in piglets (Oswald et al., 2005; Taranu et al., 2005). Mycotoxins mixtures i.e. the combinations of several micotoxis are likely to occur naturally and they may influence on the immunity in an additive or synergistic manner. Economic losses that occur as a consequence of interaction of several mycotoxins are still unknown because in low concentrations several mycotoxins may interact in way that is difficult to detect. Combinations of several and more moderate concentrations of different mycotoxins, which individually may appear to be too low in level to be a concern, can cause cumulative toxicosis, which affect the ability of the pigs organism to fight diseases (Diekman and Green, 1992; Osweiler, 2006).

On the fourth examined farm, clinically in suckling piglets the sings of severe distrurbance of the central nervous system (wide open eyes, paddling, trembling, ataxia, paresis and paralysis) were detected. In some cases the whole litter of piglets died within 48 hours.

Despite the fact that the piglets were therapeutically treated, there was no evident respond to applied medication. The pathomorphological changes that were detected in dead sucklings indicated the lesions characteristic for *Morbus Aujeszky* infection (MA) (*Necroses miliares hepatis, Haemorrhagiae corticis renis, Tonsillitis diphtheroides necroticans*). By microbiological testing in feed for lactating sows the precence of fungi (*Fusarium sp., Mucor*) and AF (0.02 mg/kg) were detected. Applying virological testing (viral isolation on the susceptible cell culture) from the tissues deriving from dead piglets the MA virus was isolated.

Aflatoxins (B1, B2, G1, G2) are recognized as immunomodulatore agents, and when AFB1 is metabolised by mammals occurs in milk as M1. It is assumed that AFB1 is the most toxic fraction (Osweiler, 2006; Živkov-Baloš et al., 2008). In the case where the outbreak of MA on the farm were examined, mycotoxin (AF) in the feed can be connected with the possible reactivation of chronioc (latent) infection. It has been discovered that aflatoxins decrease resistance to bacterial, fungal, viral and parasitic diseases in swine. Subsequently, vaccinations against various infective diseases may be less effective in animals exposed to mycotoxins (Diekman and Green, 1992). Even when is present in low doses, AF alters the immune response and this may predispose pigs to infectious diseases. From the epidemiological point of view, it is important that mycotoxins may cause breakdown of active immunity and occurence of disease even in properly vaccinated animals (Marin et al., 2002; Oswald et al., 2005; Taranu et al., 2005).

In the last examined case, in suckling piglets the occurence of neonatal diaorhea already in the first 3 days of life after farrowing were detected. These health problems did not improve after the medical treatmant with antibiotics. In great number of just farrowed piglets the most prominent clinical sign was vulvovaginitis (swelling and reddening of the vulva). Beside this, a large number of small, weak and splayleg piglets were noticed. The newborn piglets were weak, nonviable, with diarrhoea. The diseased piglets lived only for 4 days after birth. They probably died due to hypoglicemia, because sows did not have enough milk or the piglets were too weak and did not have enough strenght for milk suckling. Applying patomorphological examination on the dead suckling piglets the prominent changes on mucosal surfaces of the digestive tract (*Gastroenteritis haemorrhagica*), less number of pale kidneys, necrotic and distrophic processes on liver tissue were detected. By laboratory testing of the available swine feed grains, the presence of ZEA in different concentrations was detected (from 0.72 to 6.4 mg/kg). The ZEA mycotoxicosis in weaned piglets was clinically characterised with signs of pneumonia, slow growth, vulvovaginitis and necrosis of the tails, sporadically with diarrhoea and rectal prolapses. The patomorphological examination of the dead weaners revealed the following lesions: bleeding on the mucosal surface of the digestive tract, pleuropneumonia and pneumonia, hepatomegalia, focal nephritis and rectal prolapses. Ethiologically the pneumonia was caused by *Actinobacillus pleuropneumoniae, Haemophilus suis, and Mycoplasma hyopneumoniae.* Also the problem that was frequently observed was digestive infection with enteropathogenic *Escherichia coli*, which can be potentiated with the detected mycotoxin (ZEA 0.8 mg/kg) and a high number of different fungi species in weaners feed (*Fusarium, Penicillium, Aspergillus, Rhisopus).*

Zearalenone is a mycotoxin which as an estrogen binds competitively to estrogen receptors of the uterus, mammary gland, liver and hypothalamus (Gajecki, 2002). Pigs are the most prone to the presence and negative effects of ZEA (Diekman and Green, 1992; Obremski et al., 2003). In our research, the perinatal hyperestrogenic syndrome was a constant clinical sign in suckling piglets. This is certainly the consequence of mycotoxins presence in feed for sows, e.i. during the pregnancy and the presence of its excreted metabolite in milk of the exposed sows.

CONCLUSIONS

The influence of mycotoxins on immune system is of special interest in swine industry. The technology on swine farms demands frequent vaccinations, especially in piglets and sows which may be a problem in the case of immunocompromised animal. From the obtained

results an example of immunosuppresive effect can be presented i.e. the occurence of enteroxemia in piglets, despite the fact that dams were vaccinated twice during gestation. The enterotoxemia is caused by pathogenic bacterial strains and occurs frequently as a cause of mortality in the young categories. It can be provoked with the feed quality i. e. the presence of mycotoxins.

The presence of mycotoxins in feed can be directly connected to the detected health disturbances of the examined piglets. In our research we discovered the persistant presence of various infections, which react poorly or do not react on the applied antimicrobial therapy (gastroenteritis, pneumonia). Also, the chronic disturbances, for instance slow growth, malnutrition, vulvovaginitis suggest on the potential presence of mycotoxins.

ACKNOWLEDGEMENTS

This paper is a result of the research within the project TR 31084 " Praćenje zdravstvenog stanja divljači i uvođenje novih biotehnoloških postupaka u detekciji zaraznih i zoonoznih agenasa - analiza rizika za zdravlje ljudi, domaćih i divljih životinja i kontaminaciju životne sredine (Wild animal health monitoring and introduction of new biotechnology procedures in detection of infectious and zoonotic agents – risk analysis for human health,domestic and wild animal health and for environmental contamination)", financed by the Ministry of Education and Science, Republic of Serbia.

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FA0802: THE HUB FOR FEED FOOD AND HEALTH

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ABSTRACT: The COST Action Feed for Health has addressed several scientific topics during the last three years. From a practical point of view three main scientific fields can be identified: feed-and animal nutrition; food of animal origin (FOA) quality and functionality and consumers' perception. In this context a general scientific output of the Action is that not all FOA are the same. Milk, as a functional food has been investigated in more depth than meat and eggs. The network activities have been based on the premise that the feed selected to produce FOA on the farm determines the concentration and composition of bioactive molecules in the final product, and hence the effect on human health. FA0802 provides a unique opportunity for linking animal science with social science. From this point of view It is generally accepted that consumers prefer animal products from livestock systems that used animal feed safe for consumers, friendly to the environment and the animals. Furthermore health and sustainability are issues that show both similarities and overlap with regard to products, consumer segments and consumer behaviour, imposing to the scientific community multidisciplinary approach.

INTRODUCTION

Food of animal origin (FoA) contributes significantly to the total nutrients in the current EU diet. The latest review of livestock production and trade (FEFAC, 2010) indicates that more than 45 million tonnes of meat, and more than 135 million tonnes of milk and 7 million tonnes of eggs were produced in the EU in 2010. To sustain this scale of livestock production, about 470 million tonnes of feedstuffs are required each year within the EU-27. Clearly, ensuring such high outputs of these traded products conform to adequate quality standards is a major undertaking and it is fair to say that the EU has made significant progress in defining standards and promoting legislation in this area. As a consequence the explicit and detailed formulation of the concepts of food/feed safety and food/feed quality, has given rise, within the EU, to legislation on the traceability, control and labelling of both feed and food. However nowadays both feeds/and foods must be considered not only in terms of their nutritional properties but also in terms of their ability to promote health and protect against disease. As a consequence, the role of animal nutrition in designing foods closer to the optimum composition for long-term human health are becoming increasingly important (Feed for health MoU, 2008). Starting from these assumptions an integrated and collaborative network of research groups focused on: (i) the roles of feed and animal nutrition in improving animal health and also the quality, safety and wholesomeness of human foods of animal origin; (ii) the perception of consumers as regards the effects of feed production processes on animal health and on the quality and safety of the resulting food products, has been set up. During its lifespan the COST Action FA0802 promoted the acquisition and facilitated the dissemination of knowledge in these areas and encouraged cooperation between various research fields. Accordingly the aim of this paper is to provide an overview of the topic addressed by the Action FEED for HEALTH (www.feedforhealth.org/) using some specific examples.

NUTRITION AND HEALTH

In the field of feed-and animal nutrition the FA0802 Action provided the opportunity for addressing the impact of animal nutrition on animal health, and how to produce quality products that enhance human health whilst at the same time improving animal health. In this

context feed additives are extremely important. They contribute in general to health and welfare of the animals and provide by this a certain standard of quality of food of animal origin. Some of the nutritional additives and sensory additives have an influence on both animal wellbeing and characteristics and composition of food for human consumption, even if their formulation, dose and mode of administration can differ affecting the effectiveness (Gropp, 2010). Vitamins, vitamin-like compounds, essential fatty acids as well as probiotics and nutraceuticals are under investigation by different groups in order to evaluate their effectiveness in the animal (animal health, metabolic health, animal production efficiency) and on the quality of FoA. An example in this context is choline for dairy ruminants. Choline has been classified as vitamin-like compound, and although its requirement of dairy cows is still unknown, higher choline availability (by feeding rumen-protected choline, RPC) can have a favourable effect on milk production, especially at the onset of lactation, when choline has been proposed as limiting nutrient. Findings in transition and early lactating dairy cows in fact, suggest that greater choline availability can improve not only milk production, but also lipid, methyl group metabolism and choline secretion in milk (Pinotti et al., 2010). From a metabolic and hephatic point of view, since choline is a lipotropic factor, it may be particularly beneficial at this time in view of the adipose and liver metabolism changes that occur during the transition from late pregnancy to early lactation. Choline at this stage may optimize the balance between fat retained and fat metabolized by the liver, thereby improving lipid metabolism in general (metabolic health optimisation). Milk production response is often a consequence of these metabolic improvements.

With regard to FoA quality and functionality, the case of trace elements has been addressed in different species. For instance, as reviewed by Juniper and Bertin (2011), selenium (Se), is an essential trace element in the diets of both animals and humans; severe deficiencies have been associated with cardiomyopathies, whereas less pronounced shortfalls, although not manifesting themselves as clinical symptoms, result in sub-optimal expression of a number of Se dependent enzymes that are important for normal function and antioxidant status. Throughout much of Europe human Se intakes are well below levels needed to optimize the expression of Se dependant enzymes. A number of methods can be adopted to address this shortfall; these include the use of dietary supplements, the use of Se enhanced fertilizers or the use of dietary supplements in food producing animals diets. Animal feed supplements can contain either inorganic (sodium selenite) or organic (selenoyeast) Se. Organic sources are significantly less toxic and more bio-available than inorganic sources, principally because of differences in uptake and subsequent incorporation into animal proteins predominantly as selenomethionine (SeMet). SeMet in the body is not distinguished from methionine and is therefore actively transported across gut epithelial tissue by methionine transporter mechanisms and is either used for selenoprotein synthesis, via the selenide pathway, or incorporated non-specifically into body proteins in place of methionine. Conversely, the transport of inorganic Se across the gut epithelia is predominantly by diffusion and is then either utilised by the selenide pathway for selenoprotein synthesis or methylated and excreted. The rapid incorporation of organic Se into animal products can be demonstrated quite clearly in food producing animals, particularly in tissues and fluids that have comparatively high rates of protein inclusion or turnover such as muscle, liver and milk. In north America end in Europe milk products contribute to 25% of total selenium intake. With respect of this latter, analysis of estimates of Se intake and Se appearance in milk indicated that efficiency coefficients of transfer were greater in Selenium Yeast (SY) supplemented animals when compared to sodium selenite (SS). Furthermore, coefficients of transfer were consistent between doses in SY supplemented animals (approximately 18%) but were markedly lower in higher dose SS supplemented animals (11.3 vs. 8.1%), indicating that as SS dose increases the efficiency of total Se transfer decreases. Regression analysis of the transfer efficiency of SeMet from feed to milk indicated that changes in milk total Se concentrations appear to be more a function of the SeMet content of the animals diet rather than total Se content. It is concluded that the use of higher doses of Se supplements that have comparatively low SeMet contents would result in greater levels of Se being excreted from the animal into the environment whilst conferring little additional benefit, with respect to

shortfalls in Se intakes, to the consumers of these animal derived products (Juniper and Bertin, 2011). By contrast organic selenium administration can be useful strategy for increase its content in milk and dairy products. Similar approaches have been developd for other farm animals including fish (Moran, 2010).

In addition to the role of specific feed additives, diet formulation and in some case diet distribution can also affect quality and functionality of animal products. The case of fatty acids profile of milk and dairy products originated by cattle on pasture versus harvest forage is a further example. Milk lipids are the group of compounds which has attracted the greatest interest in research in order to change in desired directions. Further fatty acid (FA) content and composition, as well as the fat soluble vitamins are some of the compounds that are easiest to manipulate through feeding and through selection of animal breed. Main focus with respect to FA has been to increase the content of polyunsaturated fatty acids (PUFA) on expense of saturated FA. Metabolism of FA in the rumen and their transfer to milk is dependent on a great variety of factors, including accessibility of the lipid in the feed matrix, the extent of biohydrogenation in the rumen, absorption in the small intestine and transformation and utilization in the body. FA with chain length longer than C18 cannot be synthesized by the cow, therefore the occurrence and composition of these FA is highly dependent on the feed source. Typically, strategies based on lipid supplementation of high-concentrate diets are the most effective for altering milk FA composition, but these also result in significant increases in milk trans FA content. Forage based strategies, though less effective in absolute terms, have potential as a low-cost sustainable alternative, avoiding substantial increases in milk trans FA content. Thus, a detailed knowledge to the mechanisms involved in the metabolism and transference of feed lipid, metabolism in the rumen and their transference into milk as well as their interactions is of utmost importance in order to understand and control how the lipid composition of milk can be manipulated through the feeding. Likewise content of fat soluble vitamins is an important quality parameter in the milk both with respect to nutritional value and as simple antioxidants in order to protect PUFA (Savoini, 2010; Fievez, 2010; Jansen et al., 2011).

The research in dairy science has also yielded knowledge on how milk composition can be tailored to specific requirements, e.g. products targeted at risk groups, and milk for isolation and production of different bioactive molecules. Both areas are of large strategic importance for the ability of the dairy and food industry to develop novel, competitive, health-promoting products (Sejrsen personal communication). Development of nutritional strategies to improve milk composition through sustainable means can be considered as an integral component of this overall strategy. In fact, because of the wide variety of available milk products and their high consumption, these products appear as an excellent matrix for new and functional products whose consumption may have a significant impact in public health (Baldi and Pinotti, 2008).

The FA0802 Action however provides the evidence that in addition to milk, meat (McAfee, 2011) and eggs (Griffin, 2011) can contribute in designing a healthy diet. It is well established that nutritional composition of chicken feed affects the nutritional value and quality of the chicken meat and may affect chicken health. The most pronounced relationship regards fatty acids and microminerals. When the feed content of ω-3 fatty acid α-linolenic acid from oils such as linseed- and rapeseed oil is increased, the meat is enriched in these fatty acids, and a conversion towards the long ω-3 fatty acids EPA, DPA and DHA also takes place. Supplementation of organic selenium to the feed can give a meat product with a selenium concentration as high as in fish (Krogdahl, 2011). Among poultry production, it has been proven that vitamin E, carotenoids and Se are efficiently transferred to the eggs and therefore they can be easily enriched with vitamin E, Se and natural carotenoids to provide with a single egg a daily requirement in vitamin E (15 mg), 50 % RDA in Se (30-35 µg) and substantial amount of natural carotenoids (mainly lutein and zeaxanthin) (Surai, 2011).

In beef production, modern livestock breeding, production and butchery techniques have reduced the levels of fat in red meat and both livestock feeding and breed can affect the fatty acids in red meat. Industry led studies have been carried out to assess the fatty acid content of red meat, to evaluate the manipulation of fatty acid profiles in a commercial setting, to

explore breed differences and to assess consumers views on the key attributes of red meat (Maltin, 2011).
The main advantage of increasing these micro-constituents of FoA by animal nutrition rather than by postharvest fortification is that they can also safeguard the health of the animal, and this is a primary factor determining the quality, safety and wholesomeness of foods of animal origin for human consumption. Furthermore, this 'feed-to-food' approach makes it possible to reposition animal products as key foods for the delivery of important nutrients into the human diet (Baldi and Pinotti, 2008).

CONSUMERS PROSPECTIVES

Cost Action FA0802 has provided a unique opportunity for linking animal science with social science, working in the consumers perception area (figure 1).

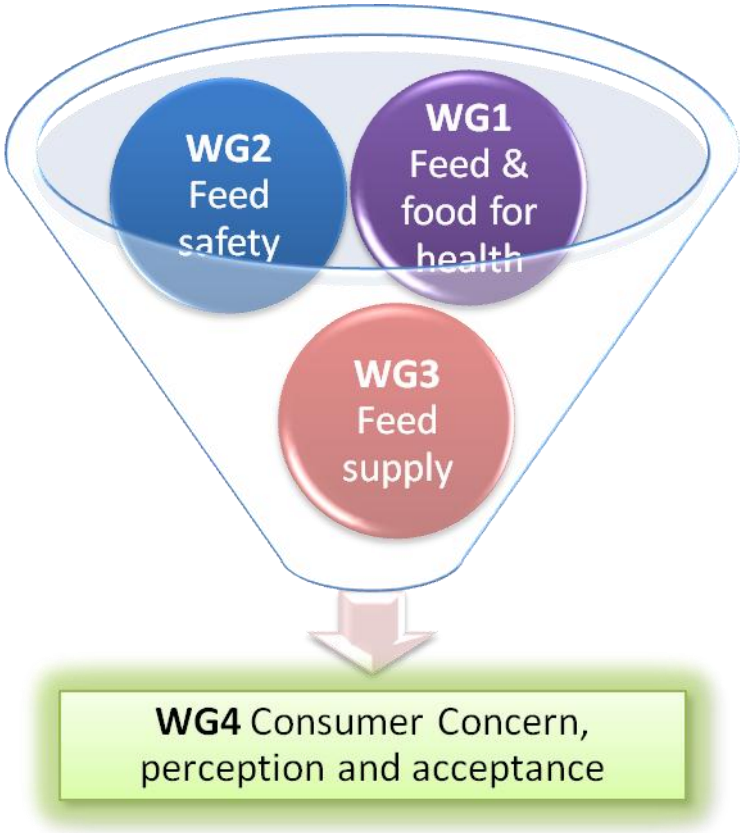

Figure 1.Feed for health Working groups

In order to address consumer expectations and concerns, it is necessary to investigate what determines consumers' food choice. Motive and value fulfilment are in general major antecedents for consumer food decision-making, and the achievement of desired consequences, such as a nice, enjoyable meal or the expected health benefits achieved by eating specific foods, are important drivers for food choices (Brunsø, 2009). However, examine the perception of consumers as regards the effects of feed production processes on animal health and on the quality and safety of the resulting food products - that consumers will be willing to purchase- is different. It is generally accepted that consumers prefer animal products from livestock systems that used animal feed safe for consumers, friendly to the environment and the animals. They also like to know that there is a traceable link of the animal feed industry to all regulations related to food safety and quality. In animal production systems, the animal feed was perceived to be particularly vulnerable to contamination, with subsequent negative impacts for the whole chain. Traceability was considered by consumers

as a useful tool that offers the potential to improve consumer confidence in food safety. Based on the consumers, the optimal livestock production is the one that would decrease feed costs, increase growth and add nutritional and ethical value. More realistically, they would prefer a balance between acceptable price and welfare conditions, with all detailed information available, but not directly to them, so as on the packaging (Luten and *Altintzoglou*, 2009; *Frewer*, 2009; *Verbeke, 2009*). All these features should match with taste. In fact, as very well addressed by Almli (2011) at the 3rd feed for health conference in Denmark, consumers do not compromise on taste for health benefits. This was the first conclusion from these studies: (i) well-accepted innovations do not alter the need for acceptable sensory properties of the product: consumers do not compromise on taste for health benefits. Furthermore (ii), from a consumer perspective, healthy innovations are highly product specific (e.g. omega-3 is not suitable for incorporation into all products), context specific (e.g. innovations of healthy character are more suitable for everyday use than for special occasion product consumption) and segment specific (e.g. rejection of pasteurisation of traditional cheese by a consumer segment in France). Finally (iii), healthy innovations may favourably be supported by appropriate consumer communication (Almli, 2011).
Thus social sciences studies suggest that public interest in livestock production practices has increased while individual perceptions have worsened following consecutive food safety crises during the last decades, combined with a growing alienation from agriculture and farming. Issues of seemingly growing concern among the broader public pertain to the environmental impact, sustainability, and animal friendliness of livestock production, as well as the intrinsic quality and safety of the resulting end products. This evolution contrasts with the actual quality of practice in contemporary livestock production in Europe (*Verbeke, 2009)*.

EMERGING ISSUES

In the COST Action feed for health we tried to address the consumers prospective about the animal production chain by involving also keynote representatives from a large retailer (e.g. Marks & Spencer plc) and food chain stake holders (e.g. Quality Meats Scotland). From their point of view feeding for health is an important topic, but its extensive application depends on efficiency, consumers demand and in turn their acceptance to pay for a premium product. Scientific and market contributions from these stakeholders to the Action has however also provided the opportunity to start a discussion about a “relatively new” topic i.e. sustainability of livestock production. Although, demand for FoA in the future could be heavily moderated by socio-economic factors, such as human health concerns and changing socio-cultural values, the global demand for livestock products is expected to double during the first half of this century, as a result of the growing human population, and its growing affluence. Over the same period, we expect big changes in the climate globally. In light of this animal production will increasingly be affected by competition for natural resources, particularly land and water, competition between food and feed and by the need to operate in a carbon-constrained economy (Thornton, 2010). For example, food, feed and fuel demand have accelerated the trend demand growth for agriculture commodities (Pinotti and Dell’Orto 2011). Among these the dramatic expansion of crop production for biofuels is already impacting on the resources available globally for food production, and hence on food supply and cost (BSAS, 2012). Moreover, the entire European food supply chain, from plant breeding, feed crop production and feed formulation, to the production of meat, dairy products, eggs, and aquaculture products, is experiencing challenges created by competition from low production cost countries and restrictions imposed by national and EU regulations on environmental impact, animal welfare and traceability. There is considerable uncertainty as to how these factors will play out in different regions of the world in the coming decades. This scenario is likely to generate new trends in the feed sector and in the feed supply chain, as recently addressed by Pinotti and Dell’Orto (2011). For example, above the competition between sectors for raw materials, the availability of by-products from bio-fuels plants, such as distillers grains and crude glycerin for use in feed, is likely to increase in the near future. This is the reason for which by-products use in animal diets formulation is matter of research worldwide, in order to

asses, above economics and marketing issues, also nutritional and safety facts and effects. One example in this field is represented by glycerol (Holtenius, 2011). It is an energy-rich byproduct from bio-diesel production that may be used as additive to diets fed to cattle. Absorbed glycerol is efficiently converted to glucose via the gluconeogenesis. Apart from the properties related to energy- and glucose metabolism glycerol also acts as substrate for *Lactobacillus reuteri* a bacterial strain with probiotic properties. Furthermore glycerol may alleviate dehydration. Glycerol of varying quality is available on the market. Crude glycerol, containing 80-90 % glycerol but also water, mineral salts and methanol, appears to currently be the quality that is most commonly used in farm animal feeding. However, diets have also been supplemented with refined glycerol, containing >99% glycerol, which is more expensive. Results from an experiment with cows in an early stage of lactation consuming crude or refined glycerol indicated that glycerol quality did not affect total dry matter intake. Obviously the bad taste and foul smell of crude glycerol did not affect the feed intake. However, intake of refined glycerol increased milk yield and the protein content in milk increased (Holtenius, 2011). Accordingly, it would be essential not only to integrate and collate knowledge on feed ingredients quality (including safety- see Feed for Health 2011 http://www.feedforhealth.org/default.asp?ZNT=S0T1O-1P58) and feed ingredients supply (market), but also to promote the acquisition and facilitate the dissemination and sharing of information between research institutions, industry, farmers and consumer organizations about feedingstuffs. Proper production and use of these by- and co-products as feed ingredients have the potential to provide both the opportunity to formulate least-cost feed, and increase significantly their value.

A different situation exists in the case of seafood. It is generally acknowledged that fish is an important part of a healthy and balanced diet. A high level of interest among European consumers in health and healthy eating, higher living standards and a good overall image of fish have yielded an increase in fish consumption. In view of that, the importance of safety and quality of fish feed, as well how consumers think about the origin of fish and aquaculture production, were key issues for the action. Aquaculture is one of the livestock sector which is growing at high rates. While most agree that fish from aquaculture is beneficial for human nutrition, concern are raised about sustainability of the raw materials used for producing fish feed (Koppe, 2010).This implies that feed supply and availability of raw materials for aqua-feed is another important issue in term of choosing sustainable and safe ingredients. The main ingredients of feeds for farmed carnivorous fish species are fish meal (FM) and fish oil (FO), at inclusion levels of about 25% and 30%, respectively. These two ingredients supply essential amino acids and fatty acids required by the fish for normal growth. Although the inclusion rates of FM nad FO in aqua-feed have been progressively reduced in the recent past (in 1985 the inclusion rate was 60% for FM, and in 2005 the level of oil was 35-40%), at present over 50% of fish meal and over 80% of fish oil produced around the world are used in aquaculture. World annual production of fishmeal and fish oil is about 6.5 million tonnes and 1.0 million tonnes, respectively from 33 million tonnes of whole fish and trimmings. Furthermore recently, small quantities of FM and FO (3-5% and 1-3%, respectively) have been included in feeds for omnivorous and herbivorous fish (Koeleman, 2009). In light of this, one of the most-frequently cited issues with the sustainable development of aquaculture is the capture of other fish as raw material to be used as fish feed in the form of fish meal and fish oil. The source of these ingredients (manufactured from wild-caught, small, bony/oily marine fish which are usually deemed not suitable for direct human consumption) in fact, are expected to remain static, or even decrease, making the supply of alternative proteins and fat sources for aqua-feed quite urgent. In this situation new development in fish nutrition find progressive role of amino acids especially taurine with conclusion that it promotes growth development and health of aquatic animals. It is believed that dietary supplementations with taurine may be beneficial for (i) increasing the chemo-attractive properties and nutritional value of aqua feeds with low fish meal inclusion. (ii) Improving fillet taste and texture (iii) enhancing immunity and tolerance to environmental stress and many more (Gupta, personal comunication). Higher doses of taurine in feed provides higher taurine contents in fish, and this can contribute to human healthy diets.

SUMMARY

Scientific results obtained across Europe in the frame of the COST Action feed for health have suggested that in general, nutritional interventions in farm animal and fish may positively affect animal health and in several cases the quality, the safety and the functionality of food of animal origin. This 'feed-to-food' approach makes it possible to reposition animal products as key foods for the delivery of important nutrients into the human diet, even though there are some distinguish between traditional and innovative food. Farther aspects addressed by the Cost action feed for heath are that health and sustainability (linked to environment and greenhouse gas emissions from farm animals) are issues that show both similarities and overlap with regard to products, consumer segments and consumer behaviour. It has been shown that consumer segments typically identified as more involved with the issue of health also tend to buy 'sustainably indicating a strong link between the two issues (Aschemann-Witzel, 2011). All these areas are of large strategic importance for the food industry to develop innovative, competitive, health-promoting products.

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POTENTIALS AND LIMITATIONS OF ENTERPENEURSHIP AMONG ACADEMIC INSTITUTION REPRESENTATIVES IN AGRO FOOD SECTOR IN SERBIA

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ABSTRACT: In innovative society as European strives to be, entrepreneurship based on innovations originating from academic research gains permanently in importance. One of the possibilities for achievement of this goal is initiation of start-ups and spin-offs by the representatives of the research community itself. Attitudes, personalities, cultural characteristics, knowledge and skills are only some of the personal features related to the probability and potential of initiation of entrepreneurial projects by representatives of academic community. The research based on the questionnaire targeted to collecting of data related to mentioned characteristics among academics involved in agro-food research was conducted was conducted in academic Institutions in Serbia. The relations among potential features of researchers and probability of initiation of enterprise ware analyzed and the most influential factors supporting entrepreneurship among academics were detected. Based on conducted research recommendations for improvement of potential for entrepreneurship among academics are provided.
Key words: *entrepreneurship, academic institutions, academics, intentions*

INTRODUCTION

In knowledge based economy universities are responsible not only for education and research process but for overall economic and social development. This new mission is transforming the traditional university into an entrepreneurial university (Lopez et al 2009). Louis et.al (1989) in pioneer research on this topic distinguished five types of academic entrepreneurship: (1) engaging in large-scale science (externally funded research), (2) earning supplemental income, (3) gaining industry support for university research, (4) obtaining patents or generating trade secrets, and (5) commercialization—forming or holding equity in private companies based on a faculty member's own research. Nowadays two commonly utilized organizational forms are recognized in relation to university engagement in entrepreneurial activities: the spin-off and the technology license agreement (Wood, 2009). According to statement of Van Burg et al. (2008) academic entrepreneurship by means of university spin-offs commercializes technological breakthroughs, which may otherwise remain unexploited. In order to foster initiation of spin offs from the universities numerous research activities in this direction have been recently realized. It has been found by Coduras et al. (2008) that entrepreneurial intention is statistically related with universities support and thus different structures have arisen in universities in order to stimulate innovation and entrepreneurial activities including spin-off companies. (Almeida 2008, Botelho and Almeida 2010) including university incubators actively established by many public universities (Todorovic and Suntorphitung 2008). It has been shown by Nossela and Grimaldi (2009) that the number of people dedicated to technology transfer activities and finally support services provided by universities have a significant influence in fostering the generation of new ventures.
Conducted research activities also prove that attitudes, knowledge, skills and expectations of academics as potential entrepreneurs play significant role for development of entrepreneurial universities. The need for the development of entrepreneurial spirit among the educated youth has been immensely recognized in developing countries and education system is

considered as a powerful tool in developing it (Jiothy, 2009) and consequently entrepreneurship programs and courses are offered by many business schools to support students and academics who aspire to start and operate own businesses.(Levenburg et al. 2006). Findings of Pilegaard et.al (2010) illustrate the importance of bridging innovation using twin skills to balance research and commercial goals, while Styhre and Lind (2010) suggest that rather than representing something radically new, the entrepreneurial university is a domain wherein traditional academic research interests and industry objectives are continuously negotiated and mutually adjusted.
Research presented in this paper was conducted with the objective to characterize the intentions towards entrepreneurship among academics from Serbia in relation to a number of personal features which can be considered as potentials or limitations for entrepreneurial ventures from academia in Serbia.

MATERIAL AND METHODS

The on line application of the questionnaire including question about intention of respondents towards entrepreneurship on one hand and questions reflecting respondents basic data, education and skills, experiences, position, incomes and personality on the other, was developed with automatic placement of data obtained from respondents into an excel data base. The questionnaire was anonymous. From the population of researchers in Serbia consisting of about 10.000 researchers the researchers who perform research which is directly or indirectly related to the agro-food sector were targeted. In order to compose the list of the researchers to be invited to fill in the questionnaire the list of research institutions accredited for research by Serbian Ministry of Education and Science and involved in national project under agro-industry and biotechnology research direction was. The list of e-mails of the researchers employed in all selected institutions was composed based on the data available on institutional web pages. Respondents were invited to fill in the questionnaire by e-mail. Limited, five day period was announced as the time during which the questionnaire is available.
The variable describing respondents intentions and attitude towards entrepreneurship was tested for independence against other collected respondents feature. Testing was performed by application of cross tabulation followed by Pearson Chi Square test of goodness of fit. Statistical software StatSoft, Inc. (2011). STATISTICA (data analysis software system), version 10. www.statsoft.com was used.

RESULTS AND DISCUSSION

Results are presented by groups of respondents' personal features including basic data and title, management experience and language skills, research area and interdisciplinary, personality and status and attitudes concerning incomes. The results are expressed as shares of respondents heaving no intentions towards entrepreneurship (inexistent intention), have been or are considering the possibility to become entrepreneurs (existent intention) or already have their own companies (realized intentions) under every examined personal feature. Results are presented in tables 1, 2, 3, 4 and 5 respectively. For each examined feature the value of p value is also presented in the tables, reflecting the probability of independence of intentions towards entrepreneurship from examined parameter.
According to the results presented in table 1 it can be stated that entrepreneurship intentions are dependent upon all examined features: gender, age and academic title with probability higher than 99%. Obtained results indicate that males are more entrepreneurship inclined confirming the findings of Rosa and Dawson (2006) and that older generation are either more entrepreneurship averse or already have their own companies, while among representatives of younger generations the share of academics who consider starting of their own companies is higher. The same observation can be applied in the case of academic title: professors and

scientist are either more entrepreneurship averse or already have their own companies, while assistants and researchers in more cases consider starting of their own companies.

Table 1. Dependence of intentions of academics towards entrepreneurship of basic personal traits

		Intentions towards entrepreneurship		
		Inexistent	Existent	Realized
Gender p=,00731	Male	35,00%	50,00%	15,00%
	Female	54,46%	40,18%	5,36%
Age p=,00146	<30	31,25%	68,75%	0,00%
	30-45	46,22%	47,90%	5,88%
	45-60	52,17%	26,09%	21,74%
	>60	46,67%	26,67%	26,67%
Title p=,00011	Professor/scientist	53,60%	31,20%	15,20%
	Assistant/researcher	38,89%	61,11%	0,00%
	Other	31,88%	65,22%	2,90%

The results presented in table 2 point out that intentions towards entrepreneurship with probability higher than 99% depend on experience in both organization and project management as well as on level of English language skills.

Table 2. Dependence of intentions of academics towards entrepreneurship of management experience and language abilities

		Intentions towards entrepreneurship		
		Inexistent	Existent	Realized
Organization management p=,00062	none	46,72%	48,91%	4,38%
	short	15,38%	53,85%	30,77%
	medium	33,33%	44,44%	22,22%
	long	60,00%	25,71%	14,29%
Project management p=,00303	none	43,94%	51,52%	4,55%
	short	25,00%	66,67%	8,33%
	medium	44,12%	29,41%	26,47%
	long	58,82%	26,47%	14,71%
English p=,00420	low	56,25%	18,75%	25,00%
	medium	65,79%	28,95%	5,26%
	high	41,61%	49,68%	8,71%

The share of academics that have no intentions towards entrepreneurship is the highest among those with long lasting experience in organization or project management. Academics with short to medium experience in organization or project management are characterized with higher share of those who already have their own companies, while academics with short management experience in most cases consider starting of the own entrepreneurial venture. Very interesting observation concerns the fact that the most academics already having their own companies have low level of English language skills, while the share of academics stating existence of intentions towards entrepreneurship increases with increase of level of English language skills.

Results presented in table 3 indicate that intentions towards entrepreneurship among academics are not dependant on the research field in which the academics are involved. The only parameter on which the dependence of intention towards entrepreneurship can be stated, with probability higher that 95 %, is the interdisciplinary orientation of the academics which includes combination of technical background (food technology, agriculture or mechanical sciences) and management or economy. Among academics with such profile the share of those without entrepreneurship intentions is the lowest while the share of those which already realized entrepreneurial ventures is the highest.

Table 3. Dependence of intentions of academics towards entrepreneurship of research area and interdisciplinary

			Intentions towards entrepreneurship		
			Inexistent	Existent	Realized
Research field	Agriculture	p=,05027	65,00%	25,00%	10,00%
	Food	p=,44572	41,35%	46,62%	12,03%
	Mechanics	p=,21217	30,61%	57,14%	12,24%
	Economy	p=,05294	39,39%	42,42%	18,18%
	Management	p=,06653	25,71%	62,86%	11,43%
	Natural	p=,60547	53,19%	36,17%	10,64%
Interdisciplinary	Technical+Economy and/or management	p=,04797	28,57%	42,86%	28,57%
	Technical + fundamental	p=,30252	50,00%	50,00%	0,00%

Table 4. Dependence of intentions of academics towards entrepreneurship of personality

		Intentions towards entrepreneurship		
		Inexistent	Existent	Realized
Imaginative p=,17749	low	72,22%	22,22%	5,56%
	average	56,60%	39,62%	3,77%
	high	38,85%	48,16%	12,99%
Creative p=,33147	low	80,00%	20,00%	0,00%
	average	56,76%	35,14%	8,11%
	high	41,40%	47,54%	11,06%
Organizer p=,62475	low	87,50%	12,50%	0,00%
	average	50,94%	41,51%	7,55%
	high	41,28%	47,16%	11,57%
Pro-active p=,01463	low	90,91%	4,55%	4,55%
	average	47,46%	50,85%	1,69%
	high	40,40%	45,97%	13,63%
Self confident p=,17764	low	58,55%	38,82%	2,63%
	average	53,13%	39,06%	7,81%
	high	37,59%	48,40%	14,00%
Communicative p=,36599	low	62,50%	31,25%	6,25%
	average	48,57%	45,71%	5,71%
	high	46,53%	42,86%	10,61%
Risk declined p=,07210	low	62,50%	31,82%	5,68%
	average	48,10%	43,04%	8,86%
	high	28,44%	56,87%	14,69%

According to the results presented in table 4 the only personality trait on which the intentions towards entrepreneurship are dependent is the the pro-active personality as already stated by Yang et al. (2006). Academics scoring this trait for themselves low in more than 90% have no intentions towards entrepreneurship.

Table 5. Dependence of intentions of academics towards entrepreneurship of attitude and position concerning personal incomes

Incomes		Intentions towards entrepreneurship		
		Inexistent	Existent	Realized
Contentment with incomes p=,12940	not at all	42,31%	34,62%	23,08%
	partly	45,61%	46,49%	7,89%
	complete	45,83%	45,83%	8,33%
Sufficiency of incomes p=,59914	much lower than needs	72,73%	18,18%	9,09%
	insufficient	39,39%	50,00%	10,61%
	sufficient	45,53%	43,90%	10,57%
	more than sufficient	50,00%	50,00%	0,00%
Willingness to increase incomes p=,25027	un conditional	40,00%	48,18%	11,82%
	conditional	52,00%	40,00%	8,00%
	inexistent	0,00%	100,00%	0,00%

In spite of expectations that the intentions towards entrepreneurship will depend upon the sufficiency, intentions and satisfaction of respondents with their incomes, obtained results, presented in table 5, indicated no dependence.

CONCLUSIONS

Based on conducted research it can be concluded that intentions towards entrepreneurial activities among academics directly or indirectly involved in agro-food related research in Serbia are more expressed under male population and among younger researchers while females and older scientists seem to be more averse towards entrepreneurship. Experience in either organization or projects management and existence of management skills especially in combination with technical skills are supportive factors regarding the intention of academics towards entrepreneurship. Satisfaction with, sufficiency off and willingness to increase incomes were not proven to influence the entrepreneurial intentions of the academics.

ACKNOWLEDGEMENT

This paper is a result of the research within the project III46001 Development and utilization of novel and traditional technologies in production of competitive food products with added value for national and global market - CREATING WEALTH FROM THE WEALTH OF SERBIA, financed by the Ministry of Education and Science, Republic of Serbia.

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ORGANIZATION OF MARKETING ACTIVITIES IN LEADER ENTERPRISE FOR PURCHASE, PROCESSING AND PLACEMENT OF NON-WOOD FOREST PRODUCTS IN VOJVODINA

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ABSTRACT: In addition to the traditional use and a range of benefits they have for local communities, non-wood forest products (NWFP) occupy a significant place in the market, particularly given the expansion of organic production and consumption. The wealth of natural diversity of Vojvodina creates an exceptional foundation for the development of entrepreneurship based on the purchase, processing and marketing of NWFP. Regardless of company size, adequately organized organizational structure, in relation to market conditions and general environment, makes one of the key determinants of efficient and effective operations. The goal pursued in this paper is to examine and analyze the organizational leadership of an enterprise system for processing and marketing of medicinal plants, primarily from the marketing aspect. Focusing on the leading companies in the industry, in Vojvodina, the research is conducted with the purpose of gaining insight into the functioning of organizational structures and ways of organizing marketing activities in it. The subject of this study was to go from the moment of purchase of raw material base and distribution of finished products. As a research technique, it is used a survey that included questions about how to organize the department in the company, personnel structure and other internal features of the company. On that basis it is formed a SWOT matrix, in which are confronted by the weak and strong companies with external opportunities and threats. The maximum capacity utilization of the developed distribution network and price competitiveness, contribute to the leading position of the surveyed company.
Key words: *organization, entrepreneurs, marketing, non-wood forest products, leader, Vojvodina*

INTRODUCTION

In recent years, domestic and international demand for non-wood forest products (NWFPs) is increasing and the forest sector gains a new awareness of their importance. NWFPs, in the broadest context, include all the biological material from forests, other than wood. In some regions they may provide a higher income in rural communities than from wood production and other traditional forest activities (2011/a). The most effective companies for processing of non-wood forest products are small, with low overheads (Nicholson et al., 2006). Majority companies in the forestry sector are small family enterprises, which is often classified as micro enterprises.[1]

One of primary determinants of effective and efficient business operations, regardless of their size is adequate organizational form compatible with the environment and the conditions prevailing in the market. According to the marketing concept, all the elements that make up the enterprise environment, external factors are considered as business for the company to provide an objective (Vasiljev et al., 2002, Vasiljev, 2005). For understanding the organization's operations, it has been interviewed the company for processing of medicinal plants which is located in Vojvodina (Figure 1).

[1] Today there are about 3000 small and medium enterprises and their jobs are based on the forest as a resource. Even 98% of all enterprises that their jobs are based on forest as resource are small enterprises (2011/b).

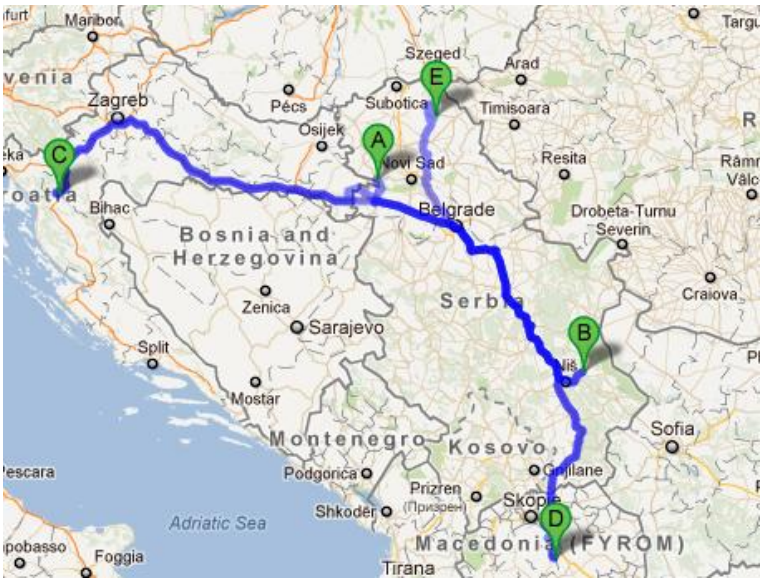

Figure 1. Map with locality of enterprise and purchase stations (Source: original)

The company is the leader in processing and marketing of medicinal plants in Vojvodina. The main **objective** of the article is analysis of the organizational structure of enterprise for the purchase, processing and placement of NWFPs. The purpose of article is analysis of functioning of organizational forms in order to consider optimal marketing strategy. The case study was the organization of company, primarily in terms of marketing activities, obtaining raw materials and distribution of final goods.

MATERIAL AND METHODS

In order to evaluate the organizational structure, which has its primary activity based on the purchase, processing and further marketing of medicinal and aromatic plants we used the survey as well as basic research techniques (2011/b). The survey includes questions about the organization sectors, particularly of marketing, the staff structure and other internal features of the company. According to the image formed on the functioning of enterprises, we created conditions for the analysis, primarily from the organizational aspects. Mutual comparison of the elements related to marketing, resulted in the constructed SWOT matrix. In that way were established strengths and weaknesses in the company, which were put in relation with external opportunities and threats. The results have created a basis for analysis, and market environment in which it operates (Schmithüsen et al., 2006).

RESULTS AND DISCUSSION

The company, which was the subject of research by the nature of the organizational type is in the company capital, in the form of joint stock company and limited liability company. The company currently has 48 permanent employees, making it, taking into account only the number of personnel by classification in the category of small enterprises. The organizational structure is based on the principle of departments, which are characteristic form of organization for small businesses. The three main divisions of the company are: procurement, sales and marketing (Diagram 1).

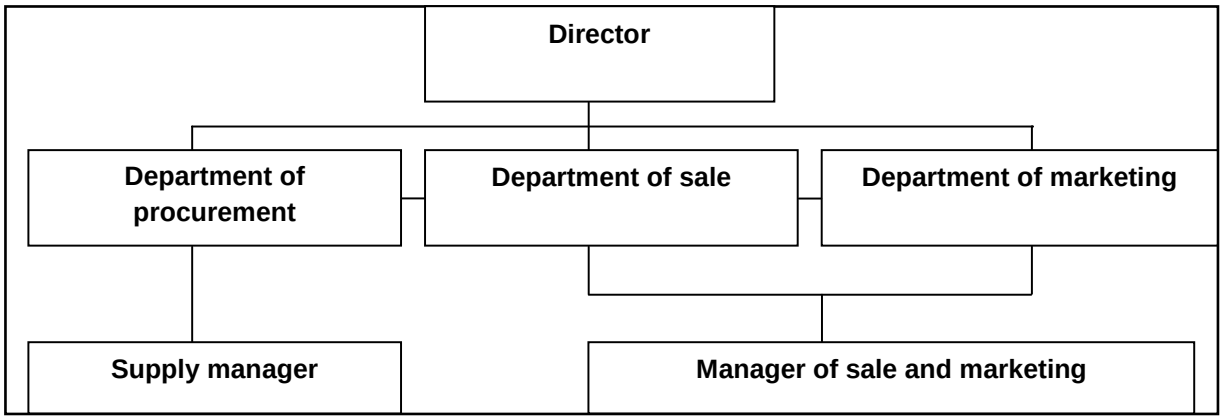

Diagram 1. The organizational structure of the company (Source: original)

Main activity of the enterprise is buying, processing and sale of medicinal plants and the company is one of the leaders in the industry. There is an active export orientation toward Western Europe and America, as well as significant coverage of domestic market with their product range. The company is a private and established in 2001. in Bačka Palanka, its activity is based on processing of medicinal plants, then their packaging and placing on the market. Oriented and to contract farming, organized the purchase of raw materials, followed by their processing and packaging operations and at the end of the final products. Their product range includes different varieties of teas in bulk and filter bags and capsules made from extracts of dried herbs. Since 2007 introduced a new technological process of production of tea in the form of granules, which is relatively innovative in this sector. The company is basically a marketing-oriented, because of its business is based on the needs and demands of consumers. In this sense, the products and the production process itself is based on the latest regulations and standards. Quality management system complies with ISO 9001:2008 and system risk analysis and control points for HACCP system. In addition, the present high level of automation in the production and application of modern technologies in the processing, packaging and finished products. Quality control of raw material base is done by accredited institutions and internally within the company.

Processing facilities for processing and packaging are part of the company and any segment is not dislocated. Supply of raw materials is carried out organized purchase of the previously agreed production. In this way, the company has security in terms of continuous supply of required quantity of raw material base suitable quality. Suppliers of raw materials are located in Svrljig and Padej, while some if it is imported from Croatia and Macedonia (Figure 2).

Transport of raw materials to processing plants is done by contract, by truck or own vans. Funds for the transport of raw materials are in accordance with the HACCP system, the part referring to the conditions to be fulfilled by means of transport for the transport of plant materials.

In the domestic market, the wholesale distribution is done independently, while sales to wholesalers, independent pharmacies and retail stores, performed by the intermediary company. Sales network is spread all over Serbia, but also present in the exporting country's environment and the Western European market. Products are completely placed to the final consumers by the intermediaries.

For activities related to procurement of raw materials in charge is manager of procurement, while business sales and marketing is managed by a person who is both a function of the manager for sales and marketing. Since the company has not fully developed the marketing department, as an independent entity, the key decisions in this field involved the entire management, through the agreement achieved at internal meetings.

The collection and sorting submitted bids is present in the marketing department. In the department actively has been coordinated with agencies for market research. Therefore, the production program of the enterprise adequately has been accommodated to the demands of

the market and consumers. In addition, there is constant cooperation with the Department of Marketing designers for making advertising and promotional materials.

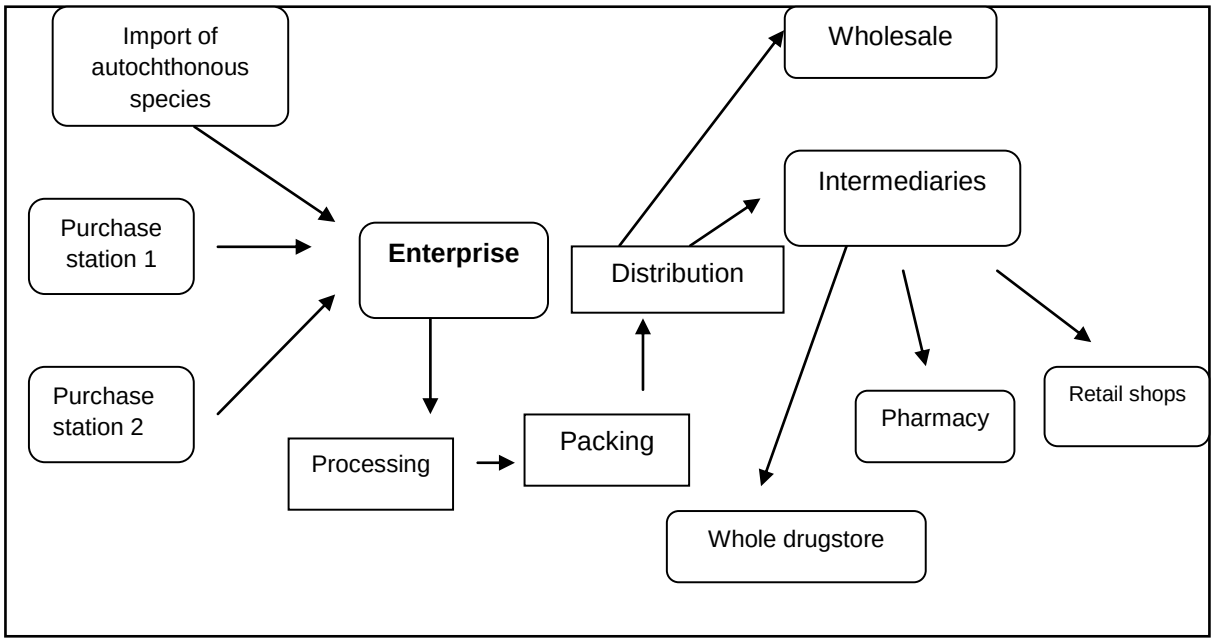

Figure 2. The supply chain of the enterprise (Source: original)

Figure 3. SWOT analysis of the enterprise

S *(strengths)*	**W *(weaknesses)***
- High product quality and price competitiveness (domestic markets)	- Strong competition in the narrow area, and insufficiently aggressive promotional activities
- A wide range of products	- Poor collection of products
- Maximum utilization of installed capacity	- Import of raw materials, causing higher costs for the company and the final products more expensive
- Accepted ISO 9001:2008 and HACCP standards	- The lack of e-business
- Wide distribution network and its own means of transport	- Lack of stuff engaged in marketing activities
- High degree of automation of production	- Department of Marketing is not fully independent
O *(opportunities)*	**T *(threats)***
- Innovative products	- Competition (with domestic and foreign markets) and the variability of prices of raw materials
-Natural Resources	
- Orientation to foreign markets	- Cheaper products of lower quality
- Expansion of processing capacities	- Low customer payment capacity
- Market presence in the "Srboflora" cluster	- Economic Crisis
- Adoption of standards and product certification	- Illiquid market
- Subsidized exports	- Influence of natural factors to the yield of medical plants

Source: original

The company promotes activities throughout magazines about healthy food, official website, and performance at various fairs and gatherings of entrepreneurs from related economic activities. Actually, the entire organization for trade shows and other events of similar type, in order to promote the product, but the whole company, was entrusted to the department of marketing. Based on the SWOT analysis (Figure 3) revealed that the company has significant internal resources. The maximum capacity utilization, with a wide range of high quality and high degree of automation in manufacturing, are its most important internalassets. Potential for development, are the innovations that are introduced into the product line, joining the National Association "Srboflora" and the increasing orientation to foreign markets. But on the other hand, there are threats which have very bad influence on the enterprise like: unfair competition and insolvent market.

According to Hegedűs et all. there is great potential around Europe for improving the market and the commercialization of NDSP, which means that in this sector there are many business opportunities in the short and long-time (Hegedus, 2007). In support of this conclusion is the fact that there is the growing demand for this group of products globally. For companies involved in purchasing, processing and marketing of NWFPs starting point is the choice of adequate suppliers, which will supply the raw materials of adequate quality at affordable prices and convenient form of payment. According to Bauch and Sills, the most obvious factor in the selection of suppliers is the price, assuming that the manufacturers choose to purchase NWFPs as raw state from manufacturers, creating a potentially higher transport costs, and potentially lower prices (Bauch and Sills, 2007). In the first case there is the case when two suppliers on the basis of purchased large amounts realized in the form of volume discount rebates. Donovan argues that SMEs in the forestry sector must overcome a number of internal weaknesses that limit their competitiveness in the globalized market. Precisely, this is a policy that is managed by a company that was the subject of the survey, because of its competitive environment exist several medium-sized companies and in this respect, emphasizing price competitiveness. Generally speaking, all companies operate on their own special system, regardless of company size or activity they are involved (Ahmetagić, 1998, Ahmetagić and Harmath, 2000).

In the studied company organizational structure is formulated in the form of classes and sectors. However, there is considerable flexibility in terms of tasks and mobility of staff within the organizational unit. The way the company is promoting its products and performs communication with the market and direct users to a large extent determines the sale itself (Vujković, 2005, Schmithüsen et al., 2006). Company as the main form of promotion uses: web site, various types of printed promotional material and active participation in trade fairs that bring together entrepreneurs from the industry. Export markets require products that are the processing procedures and comply with quality standards that are included in food products. Standardization implies a range of activities on the preparation and adoption of standards, norms and regulations. In accordance with export orientation, which is the company adopted the ISO 9001:2008[2] and HACCP[3] standards.

Because of exceptional natural predisposition of the company in terms of quality and accessibility of raw materials, in the future it will seek to expand the capacity of processing and enrichment of the product range, in line with changing market demands.

CONCLUSIONS

The company has quality raw material bases that are characteristic for organic production. All organizational units exist within their office and no segment is dislocated, which greatly contributes to more efficient operations. Export-oriented and most important quantities of

[2] The main idea and goal of ISO 9000 series of standards is to define a single system, enabling the supplier always sure that products and services meet market demands and customer needs (Acin-Sigulinski, 2002).

[3] The use of HACCP system has become a legal requirement and in Serbia under the Veterinary Act (Official Gazette RS no. 91/2005) and Law on Safety (Official Gazette RS no. 41/2009).

final products are carried in the markets of Western Europe. Products and manufacturing processes comply with ISO 9001:2008 and HACCP standards. Problems encountered in business, primarily related to competition and difficulty collecting payments due to the relatively illiquid markets and low purchasing power of consumers. Most important instruments in promoting both companies are web-sites with information and presentations for their production, printed advertising messages, advertising in magazines about healthy food and active participation in thematic exhibitions that bring together companies from segment processing of medicinal plants and other related activities. Lack of enterprise is the non-existence of an independent sector, where the required number of qualified stuff performs marketing activities. The problems faced in business, primarily related to the weak financial strength of the market. Based on the results of the SWOT matrix, the recommended alternative strategy would be **SO** (Maximum - Maximum), in which a firm should seek maximum use of internal forces, using the opportunities in the region.

ACKNOWLEDGEMENTS

This paper is a result of the research within the projects: “Održivo gazdovanje ukupnim potencijalima šuma u Republici Srbiji” (Sustainable management of total forests potential in the Republic of Serbia”) - ЕВБР 37008 and „Šumski zasadi u funkciji povećanja pošumljenosti Srbije“(Forest plantations as the indicator of increasing afforestated areas of Serbia”- ТП 31041, financed by the Ministry of Education and Science Republic of Serbia.

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CENTRAL European Congress on Food (6; 2012; Novi Sad)
Proceedings of 6th Central European Congress on Food, Novi Sad, Serbia, 23-26 May, 2012 / [editor Jovanka Lević]. – Novi Sad : Institute of Food Technology, 2012 (Novi Sad : Futura). – 1712 str. : ilustr. ; 30 cm

Tiraž 600. – Bibliografija uz svaki rad.

ISBN 978-86-7994-027-8

a) Храна – Производња – Зборници

COBISS.SR-ID 271466759